activation of Src kinase, wherein said compound decreases HBx-mediated activation of Src kinase in a cell-based assay.

53. (new) The method of claim 51 or 52 wherein said cell-based assay comprises:

a) contacting a cell expressing HBx with the compound;

b) determining the level of Src kinase activation,

wherein reduced Src kinase activation in cells expressing HBx contacted with the compound as compared to cells expressing HBx not contacted with the compound indicates that HBx-mediated activation of Src kinase has been decreased.

54. (new) The method of claim 53 wherein said compound inhibits a Src kinase signaling cascade component other than Src kinase as evaluated by an *in vitro* assay comprising:

a) contacting a Src kinase with said compound; and

b) determining the level of Src kinase activity,

wherein activity of Src kinase contacted with said compound that is not reduced as compared to activity of Src kinase not contacted with said compound indicates that said compound inhibits a Src kinase signaling cascade component other than Src kinase.

REMARKS

Claims 47-54 are pending. Claims 47-50 have been amended and new claims 51-54 has been added to more particularly point out and distinctly claim the subject matter which the Applicants regard as the invention. Support for the amended and new claims may be found in the specification. Specifically, support for claims 47-48 can be found on page 8, lines 17-23, page 17, lines 23-32, and page 19, lines 23-32 of the instant specification. Support for claims 49 and 53 can be found page 13, line 28 to page 14, line 2 and Section 5.5.1 on page 37-43 of the instant specification. Support for claims 50 and 54 can be found page 8, lines 17-23 and 34-36, page 13, line 8 to page 14, line 2, Section 5.5.1 on page 37-43 and page 13, line 28 to page 14, line 2 of the instant specification. Support for claims 51-52 can be found on page 13, line 28 to page 14, line 2, page 22, lines 12-14, page 35, lines 2-11, and Section 5.5.1 on page 37-43 of the instant specification. Thus, no new matter has been introduced.

A marked up version of the amended claims showing the amendments is attached hereto as Appendix A. Matter that has been deleted is indicated by brackets and matter that has been added is indicated by underlining. A copy of the claims as pending after entry of the foregoing amendment is attached as Appendix B. Applicants respectfully request entry of the amendments and remarks made herein into the file history of the present application.

The Rejections Under 35 U.S.C. § 112, First Paragraph Should Be Withdrawn

Claims 47-50 are rejected under 35 U.S.C. § 112, first paragraph for lack of written description. The Examiner asserts that the specification allegedly fails to provide a sufficient description of inhibitors of HBx-mediated Src kinase activity. This rejection is in error and should be withdrawn.

The specification describes numerous compounds and classes of compounds that can be used in accordance with the invention. The genus of compounds includes examples, such as tyrosine kinase inhibitors, that block the hyperphosphorylation, and therefore, activation of Src kinase.[1] In particular, the specification explicitly describes the use of several known inhibitors of Src kinase activation that, in accordance with the invention, can be used to treat HBV infection, for example, (page 7, lines 14-21 of the instant specification):

> The invention encompasses the use of known Src kinase inhibitors to treat HBV infection. Examples of such specific inhibitors include but [are] not limited to : Src specific tyrosine kinase inhibitors, such as Csk, tyrphostin-derived inhibitors, derivatives of benzylidenemalonitrile, pyrazolopyrimidine PP1, and microbial agents such as angelmicin B; and competitive inhibitors such as small phosphotyrosine containing ligands.

The written description of compounds within the claimed genus is not only explicit, but prominent. In Section 5.2.2 entitled "Compounds that Inhibits Src Kinase And Downstream Effectors of the Src Kinase Signaling Cascade," the specification not only

[1] The specification explains, by way of background, that the Src family of kinases are negatively regulated by phosphorylation of a carboxy-terminal residue. In resting cells, the Src kinases are found in a repressed state in which a carboxy-terminal tyrosine (*e.g.*, Y-527 in c-Src) is phosphorylated. Activated Src contains an additional phosphorylated tyrosine in the catalytic domain (*e.g.*, Y-416 in c-Src), which is a stimulatory event (see page 15, lines 9-36 of the instant specification). Thus, inhibition of tyrosine kinase activities involved in the hyperphosphorylation of Src kinase will inhibit Src kinase activation (see page 41, lines 9-24 of the instant specification).

specifies the use of small organic molecules that are known tyrosine kinase inhibitors that can be used to inhibit Src kinase activation, but identifies exemplary species (page 20, lines 7-20 of the instant specification):

> Examples of such tyrosine kinase inhibitors include, but are not limited to, tyrphostin-derived inhibitors, which are derivatives of benzylidenemalonitrile, have been shown to have strong inhibitory activity of Src [citation omitted], pyrazolopyrimidine PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine, a selective inhibitor of the Src family of kinases [citation omitted] and derivatives thereof. Other examples include microbial agents, such as angelmicin B, a specific inhibitor of Src tyrosine kinase activity, and derivatives thereof [citation omitted], which may also be used to inhibit HBV replication.

Here, the specification goes on to describe an entirely different class of compounds within the genus of inhibitors of Src kinase activation that could be used in accordance with the method of the invention (page 20, line 21 to page 21, line 9 of the instant specification).

> In another embodiment of the present invention, small peptides which compete with larger phosphotyrosine peptides for binding to the Src kinase protein may be used to inhibit the Src kinase signaling cascade, in particular small phosphotyrosine containing peptide ligands, 5 to 6 amino acids, which are able to compete with larger phosphotyrosine-containing peptides and protein ligands for binding to SH2 domains, thereby inhibiting the Src kinase signaling cascade and blocking replication of HBV. Another embodiment of the present invention includes small peptides which correspond to catalytic or enzymatic domains of Src kinase and would compete with Src kinase, inhibiting the activation of downstream components of the Src kinase signaling cascade. (emphasis added) Another embodiment includes the use of larger polypeptides that inhibit Src kinase activity including, but not limited to, Csk (carboxyl-terminal Src kinase) which is a specific physiologic inhibitor of Src kinase. Further examples of larger polypeptides that inhibit Src kinase activity include, for example, Src dominant-negative mutants, i.e., Srck- [citation omitted] and Fyn dominant-negative mutants [citation omitted] . . .

In view of the foregoing disclosure, it can hardly be said that members of the genus of compounds cannot be recognized! For example, Tyrphostin A9, a member of the genus of tyrphostin-derived inhibitors described in the specification, has been shown to inhibit tyrosine phosphorylation of Pyk2 (see *e.g.*, Fuortes et al., 1999, *J. Clin. Invest.* 104:327-35; Exhibit 1). This is relevant because Applicants have shown that HBx activates Pyk2 which in turns activates Src kinase (see Bouchard et al., 2001, *Science* 294:2376-8; Exhibit 2).

Thus, Tyrphostin A9 is an inhibitor of a cellular protein that is an upstream activator of Src kinase that was disclosed in the instant specification as filed and, as such, is a member of the genus of compounds described for use in the methods of the invention. Therefore, members of the genus can readily be recognized based on the disclosure. Therefore, the written description requirement is satisfied. In re Gosteli, 872 F.2d 1008, 1012, 10 U.S.P.Q.2d 1614, 1618 (Fed. Cir. 1989).

Additionally, other compounds known to those skilled in the art as potent protein tyrosine kinase inhibitors, such as staurosporine and genistein, have been shown to inhibit Pyk2 phosphorylation (see, *e.g.*, Ohmori et al., 1999, *Thrombosis Res.* 93:291-8; Exhibit 3 and Graves et al., 1997, J. *Biol. Chem.* 272:1920-8; Exhibit 4, respectively). Thus, the disclosure of the instant specification combined with what was known to one of skill in the art would have motivated evaluation of such compounds for anti-HBV activity as taught by the Applicants' disclosure. The specification need not disclose what one skilled in the art already possesses. Hirschfeld v. Banner, Commissioner of Patents and Trademarks, 200 U.S.P.Q. 276, 281 (D.D.C. 1978), aff'd, 615 F.2d 1368 (D.C. Cir. 1980), cert. denied, 450 U.S. 994 (1981). A patent application need not include in the specification that which is already known to and available to the public. Paperless Accounting Inc. v. Bay Area Rapid Transit System, 804 F.2d 659, 231 U.S.P.Q. 649 (Fed. Cir. 1986), cert. denied, 480 U.S. 933 (1987).

Claims 48 and 50 are rejected under 35 U.S.C. § 112, first paragraph for lack of enablement. The Examiner admits that the instant specification is enabling for methods of inhibiting HBV replication with compounds that reduce Src kinase activation resulting from the presence of HBx (see page 5, lines 8-13 of the instant Office Action). However, the Examiner questions enablement for inhibition of HBV replication using a compound that inhibits Src activation mediated by some non-HBx mechanism. Accordingly, without in any way conceding that this subject matter in question is not enabled by the specification and for the sole purpose of expediting prosecution of this application and with fully reserving our rights to prosecute this subject matter in a subsequent patent application, Applicants have amended claims 48 and 50. Currently pending claims 48 and 50 now specify that the compound used in the claimed methods inhibit Src kinase activity that is enhanced relative to a cell not infected with HBV. Because claim 50 depends from claim 49, it additionally specifies that the compound have activity in a cell-based assay using a

cell expressing HBx. Thus, the claims encompass subject matter that the Examiner has indicated is enabled.

In view of the foregoing, Applicants request that the Examiner withdraws the rejections under 35 U.S.C. §112, first paragraph.

The Rejection Under 35 U.S.C. § 102 Should Be Withdrawn

Claims 48-50 are rejected under 35 U.S.C. § 102(b) as being anticipated by Moriya et al., 1996, *Biochem Biophys. Res. Commun.* 218:217-223 ("Moriya"). The Examiner alleges that Moriya teaches the inhibition of HBV by administration of HBx antisense oligonucleotides. The Examiner alleges that Moriya teaches the inhibition of HBV by administration of HBx antisense oligonucleotides. Applicants respectfully disagree.

In order for a reference to anticipate a claim, each and every element of the claim must be disclosed in that one reference. Orthokinetics, Inc. v. Safety Travel Chairs, Inc., 1 U.S.P.Q.2d 1081 (Fed. Cir. 1985). "Anticipation under Section 102 can be found only if a reference shows exactly what is claimed. . . ." Structural Rubber Prod. Co. v. Park Rubber Co., U.S.P.Q. 1264 (Fed. Cir. 1984). If it is necessary to reach beyond the boundaries of a single reference to provide a missing disclosure of the claimed invention, it is not a § 102 anticipation. Scripps Clinic & Research FDN. v. Genentech Inc., 927 F.2d 1565, 18 U.S.P.Q.2d 1869 (Fed. Cir. 1991). Furthermore, anticipation is not shown even if the differences between the claims and the prior art reference are argued to be "insubstantial" and the missing elements could be supplied by the knowledge of one skilled in the art. Structural Rubber Prod. Co. v. Park Rubber Co., 221 U.S.P.Q. 1264 (Fed. Cir. 1984).

Moriya does not treat animals or subjects infected with HBV, as is required by the claims. Instead, Moriya uses a mouse model for hepatocellular carcinoma to study the effects of antisense oligonucleotides on HBx gene expression, involving mice transgenic for a single viral gene - - the HBx gene. While Moriya's animal model may be useful to evaluate hepatocellular carcinoma resulting from integration of the HBx gene into the host animal's genome - - it was not, and indeed cannot, be used to evaluate infection and viral replication - - *i.e.*, the production of progeny virions leading to viremia and chronic HBV infection.[2] Thus, Moriya does not disclose the claim limitation of a HBV-infected subject

[2] Acute viral infection causes hepatitis and other health complications. In human patients, hepatocellular carcinoma is thought to result many years subsequent to chronic

or cell, and cannot anticipate. Additionally, claims 47-48 have been amended to recite that the compound is either a small organic molecule or decreases the activity of a non-virally encoded protein. Clearly, an HBx antisense oligonucleotide meets neither of those criteria.

In the event that a reference does not explicitly teach all the elements of a claim, anticipation can only be shown by inherency if the cited reference makes clear that the missing descriptive matter is *necessarily* present in the thing described in the reference and that it would be so recognized by one of ordinary skill in the art. Continental Can Company USA, Inc. v. Monsanto Company, 948 F.2d 1264 (Fed. Cir. 1991) (emphasis added). Inherency may not be established by probabilities or possibilities. The mere fact that a certain thing *may* result from a given set of circumstances is not sufficient. In re Oelrich, 212 U.S.P.Q. 323, 326 (C.C.P.A. 1981). Substantial uncertainty regarding the existence of a product in the prior art, *i.e.*, uncertainty as to whether the inherent characteristic *necessarily* flows from the teaching of the prior art reference, is enough to preclude anticipation. W.L. Gore v. Garlock, Inc., 721 F.2d 1540, 220 U.S.P.Q. 303 (Fed. Cir. 1983; Bristol-Myers Co. v. USITC, 15 U.S.P.Q.2d 1258 (Fed. Cir. 1989).

In the present case, Moriya does not administer HBx antisense oligonucleotide to virally infected cells. Thus, Moriya does not meet each claim limitation and therefore does not constitute anticipation under § 102.

In view of the foregoing, Applicants request that the Examiner withdraws the rejection under 35 U.S.C. §102.

The Rejection Under 35 U.S.C. § 103 Should Be Withdrawn

Claims 47 and 49-50 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Moriya. The Examiner alleges that Moriya teaches the inhibition of HBx gene transcription and translation by administration of HBx antisense oligonucleotides. As such, the Examiner contends that administration of the antisense oligonucleotide would inherently inhibit HBx-mediated Src kinase activation and thus fall within the scope of the claims. Applicants respectfully disagree and point out below that the currently pending claims are not rendered obvious by the disclosures of the cited reference.

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infection by HBV, as a consequence of integration of parts of the HBV genome into the genome of the host liver cells.

Preliminarily, Applicants point out that claims 47-50 relate to methods for inhibiting HBV infection or replication in an HBV-infected subject or cell with a compound that decreases the activity of a non-virally encoded protein such that Src kinase activity that is enhanced relative to an uninfected subject or cell is inhibited. Newly added claims 51-54 relate to methods for inhibiting HBV infection or replication in an HBV-infected subject or cell with a therapeutically effective amount of a compound that decreases HBx-mediated activation of Src kinase in a cell-based assay.

According to the Examiner, "While Moriya et al. do not show that these oligonucleotides inhibit activation of Src kinase, this is an inherent effect of the antisense oligonucleotide of Moriya et al. as the oligonucleotide of Moriya inhibited HBx expression such that there is no HBx present to activate Src kinase." (page 9 of the instant Office Action). Applicants point out that the Examiner is improperly using inherency as a basis for an obviousness rejection. This is legally improper. Inherency is immaterial in an obviousness analysis if the record establishes that one of ordinary skill in the art would not appreciate or recognize the inherent feature. In re Shetty, 566 F.2d 81, 195 U.S.P.Q. 753 (C.C.P.A. 1977). As stated by the CCPA in In re Spormann, "[t]hat which may be inherent is not necessarily known. Obviousness cannot be predicated on what is unknown." In re Spormann, 363 F.2d 444, 448, 150 U.S.P.Q. 449, 452 (C.C.P.A. 1966); see also W.L. Gore & Assocs., Inc. v. Garlock, Inc., 721 F.2d 1540, 1555, 220 U.S.P.Q. 303, 314-15 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984).

Applicants submit that there is no evidence that the skilled artisan would have appreciated or recognized that HBx antisense oligonucleotides would inhibit Src kinase activation. Moriya administered HBx antisense oligonucleotides to HBx-expressing transgenic mice in an effort to probe the connection between HBx expression and hepatocellular carcinoma pathogenesis. Moriya did not measure the expression or activation level of any non-viral cellular protein. The sole parameter assayed was the degree of lesioning in the liver. Moriya did not elucidate, or even attempt to elucidate, which pathway HBx was working through to elicit the liver pathology. As such, the involvement of Src kinase in the signaling pathway was completely unappreciated. One of skill in the art, upon reading Moriya's disclosure, would not be motivated to use compounds that inhibit Src kinase activation to treat HBV-infected patients or to decrease HBV replication.

A finding of obviousness under 35 U.S.C. § 103 requires a determination of the scope and the content of the prior art, the differences between the invention and the prior art, the level of the ordinary skill in the art, and whether the differences are such that the claimed subject matter as a whole would have been obvious to one of ordinary skill in the art at the time the invention was made. Graham v. Deere, 383 U.S. 1 (1966). The relevant inquiry is whether the prior art suggests the invention, and whether one of ordinary skill in the art would have had a reasonable expectation that the claimed invention would be successful. In re O'Farrell, 853 F.2d 894, 902-4 (Fed. Cir. 1988); In re Vaeck, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed. Cir. 1991). Both the suggestion of the claimed invention and the expectation of success must be in the prior art, not in the disclosure of the claimed invention. In re Dow Chemical Co., 5 U.S.P.Q. 2d 1529 (Fed. Cir. 1988).

The currently pending claims require that the compound to be used in the methods of the invention to be a compound that i) is a small organic molecule that inhibits Src kinase activity that is enhanced relative to a subject/cell not infected with HBV, ii) decreases the activity of a non-virally encoded cellular protein, or iii) decreases HBx-mediated activation of Src kinase in a cell based-assay. Applicants submit Moriya not only fails to disclose such a compound, but does not even render the use of such a compound to inhibit HBV infection/replication obvious.

Firstly, the requirement that a compound used in the methods of the invention be a compound that is a small organic molecule that inhibits Src kinase activity that is enhanced relative to a subject/cell not infected with HBV has not been suggested. Moriya proposes the use of HBx antisense oligonucleotides as a method of preventing hepatocellular carcinoma in HBV infection - - not as a means of inhibiting HBV infection/replication. One skilled in the art would view hepatocellular carcinoma as fundamentally different from HBV virus infection. This is especially true of the hepatocellular carcinoma described in Moriya which was the result of artificial high level recombinant expression of one viral protein in transgenic mice. It was not until Applicants' disclosure of HBx activation of Src kinase and the indispensable role Src kinase activation plays in HBV infection/replication that it would be obvious to one of skill in the art to use a small organic molecule that inhibits Src kinase activity that is enhanced relative to a subject/cell not infected with HBV.

Secondly, the requirement that a compound used in the methods of the invention be a compound that decreases the activity of a non-virally encoded cellular protein has not been suggested. As discussed above, Moriya did not have any indication as to the cellular

mechanism through which HBx acted to effect liver pathology. Because no non-virally encoded protein was known to be involved, one skilled in the art would have no suggestion as to what cellular protein to target for inhibition in order to decrease HBV replication/inhibition. No evidence or reference has been provided by the Examiner to remedy this defect in Moriya's disclosure. In fact, it is the Applicants' own invention that provides the crucial link between HBx and Src kinase.

Thirdly, the requirement that a compound used in the methods of the invention be capable of inhibiting HBx-mediated Src kinase activity in cell-based assay has not been suggested. An antisense oligonucleotide that inhibits expression of HBx cannot decrease the enhanced Src kinase activation that results *in the presence* of HBx. In fact, the Examiner points out that HBx transcription and translation appear to be completely inhibited in the transgenic mice upon treatment with the antisense oligonucleotide (page 8 of the instant Office Action). Moriya's teachings, therefore, give no suggestion of how to inhibit Src activation while HBx is expressed and present. Applicants' discovery that HBx activated the Src kinase signaling pathway during HBV infection/replication was the first suggestion of a cellular target that could effect HBV inhibition.

Applicants submit that rejection of the instant claims under § 103 is an improper use of hindsight gained from Applicants' own specification. Hindsight should be avoided in applying the nonobviousness requirement. Panduit Corp. v. Dennison Mfg. Co., 810 F.2d 1561, 1 U.S.P.Q.2d 1593 (Fed. Cir. 1987), cert. denied, 481 U.S. 1052 (1987). "One cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention." In re Fine, 837 F.2d 1071, 1075, 5 U.S.P.Q.2d 1596, 1600 (Fed. Cir. 1988).

Without the benefit of hindsight, the teaching of the reference cited by the Examiner could not possibly render obvious the claimed invention. The claimed methods could not have been foreseen by a person of ordinary skill in the art since there was no suggestion of them in the art. A finding of obviousness could only be arrived through a prohibited procedure in which "the claims were used as a frame, and individual naked parts of separate prior art references were employed as a mosaic to recreate a facsimile of the claimed invention." W.L. Gore & Assocs. Inc. v. Garlock, Inc., 721 F.2d 1540, 1552, 220 U.S.P.Q. 303, 312 (Fed. Cir. 1983), cert. denied, 469 U.S. 851 (1984).

In view of the foregoing, Applicants request that the Examiner withdraws the rejection under 35 U.S.C. §103.

CONCLUSION

Applicants respectfully request that the amendments and remarks made herein be entered and made of record in the file history of the present application. Withdrawal of the Examiner's rejections and a notice of allowance are earnestly requested. If any issues remain in connection herewith, the Examiner is respectfully invited to telephone the undersigned to discuss the same.

Respectfully submitted,

Date: April 22, 2003

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APPENDIX A
MARKED VERSION OF THE AMENDED CLAIMS
U.S. PATENT APPLICATION SERIAL NO. 09/096,589
ATTORNEY DOCKET NO. 5914-065-999

47. (amended) A method for inhibiting Hepatitis B virus (HBV) infection or replication comprising administering a compound to an HBV-infected patient that inhibits [enhanced activity of] Src kinase activity that is enhanced relative to a patient not infected with HBV, wherein said compound is a small organic molecule or decreases the activity of a non-virally encoded cellular protein [enhanced activity results from the presence of HBx].

48. (amended) A method for inhibiting Hepatitis B virus (HBV) infection or replication in a cell [wherein Src kinase activity is enhanced] comprising [administering] contacting a HBV-infected cell with a compound that [reduces the enhanced activation of] inhibits Src kinase activity that is enhanced relative to a cell not infected with HBV, wherein said compound is a small organic molecule or decreases the activity of a non-virally encoded cellular protein [to levels comparable to those observed in the absence of HBV].

49. (amended) The method of Claim 47 or 48 wherein [the] said compound that inhibits [said enhanced activity of] said Src kinase activity that is enhanced is [determined] evaluated by an *in vitro* assay comprising:

a) contacting a cell expressing HBx with the compound;

b) determining [whether levels] the level of Src kinase activity,

wherein reduced Src kinase activity in cells expressing HBx contacted with said compound as compared to cells expressing HBx not contacted with said compound indicates that enhanced Src kinase activity has been inhibited

[are reduced in those cells contacted with the compound as compared to levels of Src kinase activity in cells expressing HBx in the absence of the compound].

50. (amended) The method of claim [47 or 48] 49 wherein said compound inhibits a Src kinase signaling cascade component other than Src kinase as [determined] evaluated by an *in vitro* assay comprising:

a) contacting a Src kinase with said compound; and

b) determining [whether levels] the level of Src kinase activity,

wherein activity of Src kinase contacted with said compound that is not reduced as compared to activity of Src kinase not contacted with said compound indicates that said compound inhibits a Src kinase signaling cascade component other than Src kinase [in the presence of said compound are substantially equal to levels of Src kinase activity in the absence of said compound].

APPENDIX B
PENDING CLAIMS AS OF APRIL 22, 2003
U.S. PATENT APPLICATION SERIAL NO. 09/096,589
ATTORNEY DOCKET NO. 5914-065-999

47. (amended) A method for inhibiting Hepatitis B virus (HBV) infection or replication comprising administering a compound to an HBV-infected patient that inhibits Src kinase activity that is enhanced relative to a patient not infected with HBV, wherein said compound is a small organic molecule or decreases the activity of a non-virally encoded cellular protein.

48. (amended) A method for inhibiting Hepatitis B virus (HBV) infection or replication in a cell comprising contacting a HBV-infected cell with a compound that inhibits Src kinase activity that is enhanced relative to a cell not infected with HBV, wherein said compound is a small organic molecule or decreases the activity of a non-virally encoded cellular protein.

49. (amended) The method of Claim 47 or 48 wherein said compound that inhibits said Src kinase activity that is enhanced is evaluated by an *in vitro* assay comprising:

a) contacting a cell expressing HBx with the compound;

b) determining the level of Src kinase activity,

wherein reduced Src kinase activity in cells expressing HBx contacted with said compound as compared to cells expressing HBx not contacted with said compound indicates that enhanced Src kinase activity has been inhibited.

50. (amended) The method of claim 49 wherein said compound inhibits a Src kinase signaling cascade component other than Src kinase as evaluated by an *in vitro* assay comprising:

a) contacting a Src kinase with said compound; and

b) determining the level of Src kinase activity,

wherein activity of Src kinase contacted with said compound that is not reduced as compared to activity of Src kinase not contacted with said compound indicates that said compound inhibits a Src kinase signaling cascade component other than Src kinase.

51. (new) A method for treating HBV infection, comprising administering to an HBV-infected patient a therapeutically effective amount of a compound that inhibits activation of Src kinase, wherein said compound decreases HBx-mediated activation of Src kinase in a cell-based assay.

52. (new) A method for inhibiting HBV replication comprising contacting an HBV-infected cell with a therapeutically effective amount of a compound that inhibits activation of Src kinase, wherein said compound decreases HBx-mediated activation of Src kinase in a cell-based assay.

53. (new) The method of claim 51 or 52 wherein said cell-based assay comprises:

a) contacting a cell expressing HBx with the compound;

b) determining the level of Src kinase activation,

wherein reduced Src kinase activation in cells expressing HBx contacted with the compound as compared to cells expressing HBx not contacted with the compound indicates that HBx-mediated activation of Src kinase has been decreased.

54. (new) The method of claim 53 wherein said compound inhibits a Src kinase signaling cascade component other than Src kinase as evaluated by an *in vitro* assay comprising:

a) contacting a Src kinase with said compound; and

b) determining the level of Src kinase activity,

wherein activity of Src kinase contacted with said compound that is not reduced as compared to activity of Src kinase not contacted with said compound indicates that said compound inhibits a Src kinase signaling cascade component other than Src kinase.

Exhibit 1

Rol of the tyrosine kinase pyk2 in th int grin-dependent activation of human n utrophils by TNF

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Secretion of inflammatory products from neutrophils can be induced by a combination of signals from ligated integrins and receptors for soluble, physiological agonists such as TNF. Here we identify pyk2 in primary human neutrophils; localize it to focal adhesions and podosomes; and demonstrate its tyrosine phosphorylation, activation, and association with paxillin during stimulation of adherent cells by TNF. Tyrphostin A9 emerged as the most potent and selective of 51 tyrosine kinase inhibitors tested against the TNF-induced respiratory burst. Tyrphostin A9 inhibited TNF-induced tyrosine phosphorylation of pyk2 without blocking the cells' bactericidal activity. Wortmannin, an inhibitor of phosphatidylinositol-3-kinase, potently blocked the TNF-induced respiratory burst and selectively inhibited tyrosine phosphorylation of pyk2. Thus, pyk2 appears to play an essential role in the ability of neutrophils to integrate signals from β_2 integrins and TNF receptors.

J. Clin. Invest. 104:327–335 (1999).

Introduction

Secretion of microbicidal and histotoxic molecules by polymorphonuclear leukocytes (PMNs) stimulated by soluble, physiological agonists depends on the cells receiving 2 signals (1). Ligation of integrins informs the cells that they have made multiple contacts with extracellular matrix proteins in the tissues. The second signal is delivered through receptors for inflammatory cytokines, chemokines, eicosanoids, glyceryl ethers, formyl peptides, or activated complement. These responses not only underlie antimicrobial defense but contribute to tissue damage in such states as septic shock, respiratory distress syndrome, ischemia-reperfusion, and rheumatoid arthritis. Thus, understanding how PMNs integrate the components of the binary signals controlling their activation may hold a key to new anti-inflammatory therapies.

TNF is a powerful mediator of the innate immune response. Despite intense interest in TNF signaling, it remains unclear how TNF activates PMNs. Most studies of signal transduction through TNF receptors have dealt with gene expression, cell proliferation, or apoptosis, mostly in transformed cell lines. In primary PMNs, however, TNF elicits spreading, exocytosis, and a respiratory burst independently of transcription and translation (2). No role in these responses has been described for any of the proteins thus far known to be associated with the intracellular domains of TNF receptors.

In vitro, TNF-treated PMNs spread on matrix protein-coated surfaces (3) and tyrosine phosphorylate several focal adhesion proteins (4, 5). The latter include paxillin (4, 6) and the tyrosine kinases fgr (7), lyn (8), and syk (9). When spreading is advanced, the cells abruptly begin to release H_2O_2 and granule contents (10) at a maximal rate (2, 3).

Protein tyrosine phosphorylation is necessary for the respiratory burst of adherent PMNs (5). PMNs from mice rendered genetically deficient in either of 2 src-family tyrosine kinases, fgr or hck, responded to TNF normally, but PMNs from mice doubly deficient in fgr and hck neither spread nor secreted oxidants in response to TNF (11). However, further studies strongly suggested that activation of fgr is not essential for the integrin- and TNF-dependent respiratory burst in human PMNs. On the contrary, activation of fgr and lyn in human PMNs appears to be a consequence, rather than a cause, of the respiratory burst (12). As yet, there has been no identification of any specific tyrosine kinase whose activity is required for the cytokine-induced, adhesion-dependent respiratory burst of human PMNs.

Tyrosine phosphoproteins in TNF-stimulated, adherent human PMNs were localized to punctate structures on the adhering surface containing vinculin, a marker of focal adhesions (6). This observation directed our attention to tyrosine kinases associated with such structures. Among the tyrosine kinases localized to focal adhesions and involved in integrin signaling are the focal adhesion kinases, whose prototype is FAK (13). FAK is activated after β_1 and β_3 integrin stimulation in fibroblasts and platelets and binds β_1 and β_3 integrins, src, CSK, paxillin, GRAF, Cas, phosphatidylinositol 3-kinase (PI3K), and sos/Grb2 (reviewed in ref. 14). Human PMNs contain FAK, but its tyrosine phosphorylation

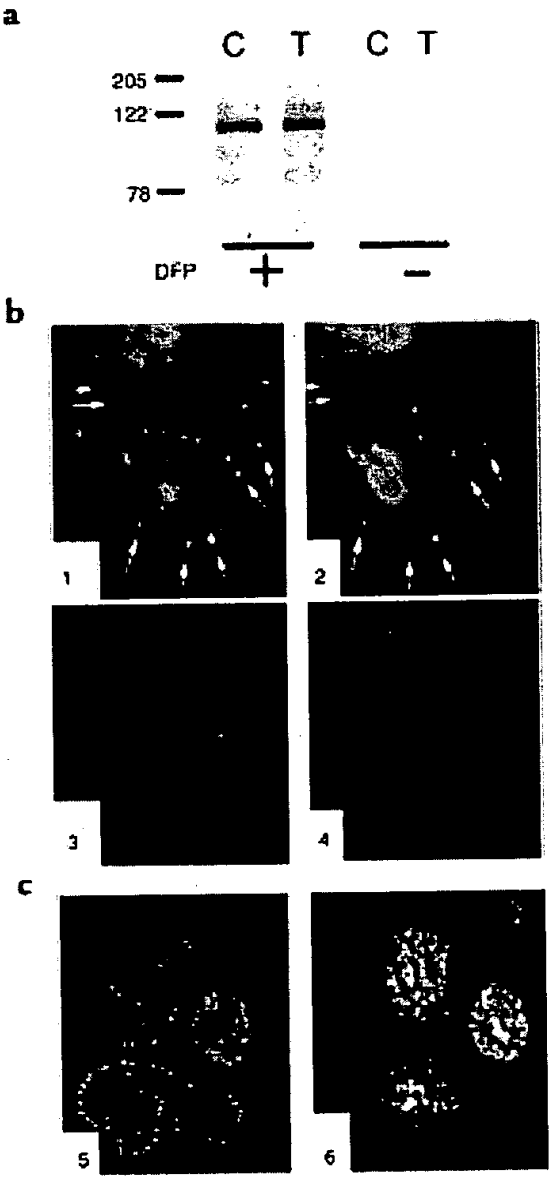


Figure 1
Identification of pyk2 in human PMNs and its localization to adhesion structures. (**a**) Pyk2 is evident only in PMNs treated with diisopropylfluorophosphate before lysis (first 2 lanes), in addition to the other protease inhibitors used in the second 2 lanes (see text). PMNs were plated on FBS-coated plates and treated with buffer alone (control; C) or TNF (250 ng/mL; T) for 60 minutes. Identical amounts of protein (100 μg) were loaded in each lane. (**b**) Indirect immunofluorescence. PMNs adherent to FBS-coated glass coverslips were stimulated with TNF (100 ng/mL) for 60 minutes, fixed, permeabilized, and stained with rabbit anti-pyk2 antibody (panel 1) or mouse anti-vinculin mAb (panel 2), followed by Cy3-conjugated donkey anti-rabbit (panels 1 and 3) or Cy2-conjugated donkey anti-mouse IgG (panels 2 and 4). In panel 3, primary antibody was omitted. In panel 4, an irrelevant mAb was used. The photomicrograph is focused at the plane of cell contact with the substratum. (**c**) Confocal microscopy of cells prepared as in **b** and stained with rabbit anti-pyk2 antibody (panel 5) or mouse anti-vinculin mAb (panel 6). Sum of four 0.32-μm-thick images taken from the plane of contact with the substratum.

is not altered by adhesion (6, 15) or TNF stimulation (6), although spontaneous spreading on laminin promotes it (15).

Recently, a second member of the FAK family has been identified, termed pyk2 (proline-rich tyrosine kinase) (16), CAKβ (17), and RAFTK (18). Pyk2 is more prominent than FAK in unseparated peripheral blood leukocytes. Although pyk2 and FAK are highly homologous, the amino acid sequences surrounding the conserved tyrosines are not identical, suggesting that pyk2's binding partners, including the src kinases that activate it, may be different than FAK's. Indeed, in cells possessing both kinases, one but not the other is activated by a given stimulus (19, 20). Pyk2 is activated by TNF in HL-60 leukemic cells (21) but has not been identified in primary PMNs.

The present study demonstrates pyk2 in primary human PMNs; localizes it to focal adhesions and podosomes; and demonstrates its tyrosine phosphorylation, activation, and association with paxillin during activation of the cells by TNF. Of 51 chemically distinct tyrosine kinase inhibitors tested, 1 was a particularly potent and selective inhibitor of the TNF-induced respiratory burst. This agent, tyrphostin A9, also inhibited the tyrosine phosphorylation of pyk2. Wortmannin, a PI3K inhibitor, also blocked pyk2 phosphorylation, while leaving most tyrosine phosphorylation intact. Thus, pyk2 appears to play an essential role in the ability of PMNs to integrate signals from β_2 integrins and TNF receptors, and participates in a signal cascade of interest as a potential target for anti-inflammatory therapy.

Methods

PMNs and test agents. Human PMNs were isolated from heparinized blood of normal donors and processed as described for immunoprecipitation (6), immunoblot (6), microscopy (1), H_2O_2 release (3), and bactericidal function (22). Pure recombinant human TNF was a gift of Genentech Inc. (South San Francisco, California, USA) PMA was from Sigma Chemical Co. (St. Louis, Missouri, USA). Tyrosine kinase inhibitors and wortmannin were obtained from Calbiochem-Novabiochem Corp. (San Diego, California, USA) or from Samuel Wright at Merck Research Laboratories (Rahway, New Jersey, USA), where they were assembled from commercial sources.

Immunoprecipitation, immunoblot, and immunocytochemistry. Rabbit anti-pyk2 antibody was a gift of H. Avraham (Harvard Institute of Medicine, Boston, Massachusetts, USA) (18) and was also purchased from Upstate Biotechnology Inc. (Lake Placid, New York, USA). Goat anti-PYK2 was from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Rabbit anti-phosphotyrosine antibody, mouse anti-phosphotyrosine mAb (PY20-IgG2b), recombinant mouse anti-phosphotyrosine mAb coupled to horseradish peroxidase (RC20), and anti-paxillin (349-IgG1) mAb were from Transduction Laboratories

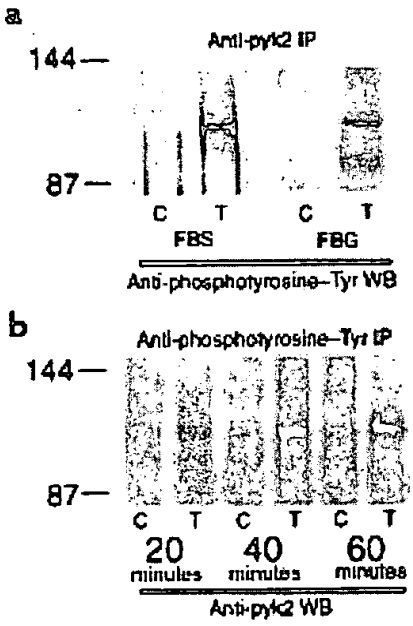


Figure 2
TNF-stimulated tyrosine phosphorylation of pyk2. (a) PMNs were plated on fetal bovine serum–coated (FBS) or fibrinogen-coated (FBG) plates and treated with buffer alone (control; C) or TNF (250 ng/mL; T) for 60 minutes (FBS) or 30 minutes (FBG). Cell lysates (200 µg) were immunoprecipitated (IP) with anti-pyk2 goat antibody. Proteins were separated by reducing SDS-PAGE, transferred to nitrocellulose, and Western blotted (WB) with horseradish peroxidase–conjugated (HRP-conjugated) anti-phosphotyrosine mAb followed by enhanced chemiluminescence (ECL) detection. (b) PMNs were plated on FBS-coated plates and treated with buffer alone (control; C) or TNF (250 ng/mL; T) for 20, 40, or 60 minutes as indicated. Cell lysates (500 µg) were immunoprecipitated (IP) with anti-phosphotyrosine mAb. Proteins were separated by reducing SDS-PAGE, transferred to nitrocellulose, and Western blotted (WB) with anti-pyk2 antibody followed by ECL detection. Molecular mass markers are indicated in kilodaltons.

(Lexington, Kentucky, USA). Anti-vinculin mAb (hVIN-1-IgG1) was from Sigma Chemical Co.

Immunoprecipitation and immunoblot were as reported (6), including pretreatment with 5 mM diisopropylfluorophosphate, except that for coimmunoprecipitation, cells were lysed in 200 µL of nondenaturing modified RIPA buffer (10 mM Tris-HCl [pH 7.5], 1% Triton X-100, 150 mM NaCl, 1 mM sodium vanadate, 0.1 mM sodium molybdate, 1 mM sodium pyrophosphate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 5 µg/mL each of pepstatin A, leupeptin, aprotinin, and chymostatin) on ice for 30 minutes. A Bio-Rad MRC600 laser scanning confocal microscope (Bio-Rad Microscience, Cambridge, Massachusetts, USA) with 2 photomultiplier tubes was used on an inverted Zeiss Axiovert microscope (Zeiss, Oberkochen, Germany) with a ×63 (NA 1.4) Zeiss Planapo objective for simultaneous detection of Cy2- and Cy3-conjugated antibodies. Images were processed with Metamorph software (Universal Image Corp., West Chester, Pennsylvania, USA).

Kinase assay. After immunoprecipitation with goat or rabbit anti-pyk2 antibodies, immune complexes were further washed in kinase buffer (25 mM HEPES, 1 mM DTT, 10 mM $MnCl_2$, 5 µM ATP [pH 7.4]) and then incubated for 30 minutes at room temperature in kinase buffer supplemented with [γ-^{32}P]ATP (0.1 µCi, 10 Ci/mmol). Proteins were separated on SDS-PAGE gels, transferred electrophoretically to PVDF membrane, and autoradiographed.

Results

Identification and localization of pyk2 in human PMNs. Pyk2 was detected by immunoblot in preparations containing more than 98% PMNs, but only when the cells were treated before cell lysis with diisopropylfluorophosphate. Other protease inhibitors (phenylmethylsulfonyl fluoride, pepstatin A, leupeptin, aprotinin, and chymostatin in combination) were unable to preserve pyk2 (Figure 1a). Thus, protease sensitivity may explain why pyk2 was not identified previously in primary PMNs.

In adherent PMNs, phosphotyrosine-containing proteins are predominantly localized in punctate podosomes (23) abutting the cell surface in contact with the subjacent matrix. The distribution of pyk2 in primary human PMNs was consistent with localization both to podosomes and to oblong structures at the cell periphery, typical of focal adhesions (Figure 1, b and c). Pyk2 colocalized with vinculin, a component of focal adhesions. Pyk2 in both sets of structures was confined to the cell surface in contact with the substratum (Figure 1b), as confirmed by confocal microscopy (Figure 1c).

TNF-dependent tyrosine phosphorylation of pyk2. Stimulation of adherent PMNs with TNF causes a time-dependent increase in tyrosine phosphorylation of proteins with an apparent M_r of ~120 kDa, compatible with pyk2 (5). When PMNs adherent to fibrinogen-coated or FBS-coated plates were stimulated with TNF for 60 minutes, immunoprecipitation with anti-pyk2 followed by immunoblot with anti-phosphotyrosine antibody demonstrated TNF-dependent tyrosine phosphorylation of pyk2 (Figure 2a). Similar amounts

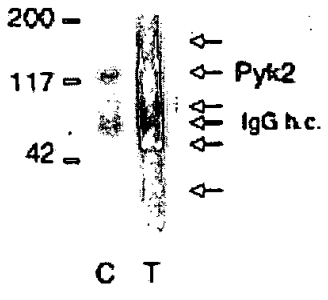


Figure 3
Activation of pyk2 by TNF. PMNs plated on FBS-coated dishes were treated with buffer alone (control; C) or TNF (250 ng/mL; T) for 60 minutes. PMNs were lysed in modified RIPA buffer. Lysates (200 µg) were immunoprecipitated with anti-pyk2 antibody, and the immunoprecipitates were subjected to in vitro kinase assay, separated on reducing SDS-PAGE, transferred to a nylon membrane, and autoradiographed. Prominent species are marked by arrows, including IgG heavy chain (h.c.). Molecular mass markers are indicated in kilodaltons.

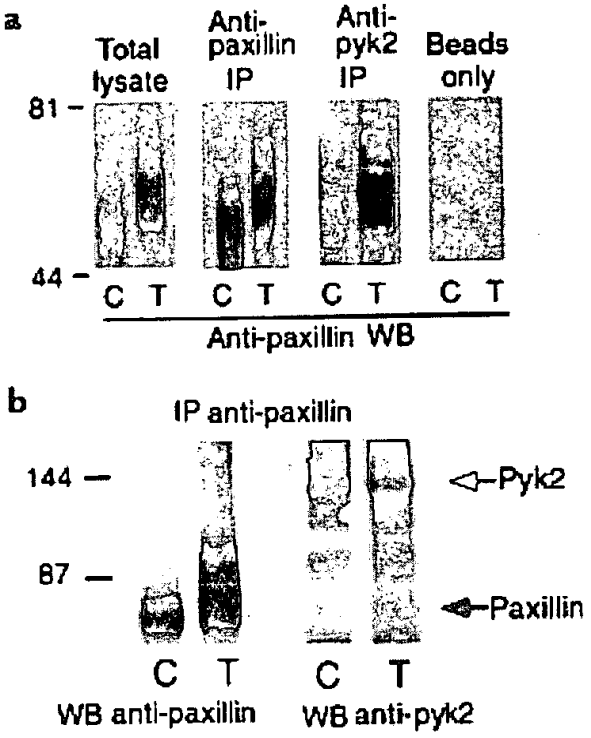


Figure 4

TNF-enhanced association of pyk2 with paxillin. (a) Coimmunoprecipitation of paxillin with pyk2. PMNs plated on FBS-coated dishes were treated with buffer alone (control; C) or TNF (250 ng/mL; T) for 60 minutes. PMNs were lysed in modified RIPA buffer. Lysates were immunoprecipitated (IP) with the indicated antibodies or with protein A-Sepharose beads alone, separated on reducing SDS-PAGE, transferred to nitrocellulose, and Western blotted (WB) with anti-paxillin mAb followed by anti-mouse HRP-conjugated antibody with ECL detection. Molecular mass markers are indicated in kilodaltons. (b) Coimmunoprecipitation of pyk2 with paxillin. PMNs plated on FBS-coated dishes were treated with buffer alone (control; C) or TNF (250 ng/mL; T) for 60 minutes and lysed in modified RIPA buffer. Lysates were immunoprecipitated with anti-paxillin mAb, separated on reducing SDS-PAGE, transferred to nitrocellulose, and Western blotted (WB) with anti-paxillin mAb or anti-pyk2 antibody followed by anti-mouse or anti-goat HRP-conjugated antibodies with ECL detection. Molecular mass markers are indicated in kilodaltons.

of pyk2 were precipitated in all samples (data not shown). No tyrosine phosphorylation of pyk2 was detected in suspended PMNs treated or not with TNF (data not shown). TNF-dependent tyrosine phosphorylation in adherent PMNs was shown to involve β_2 integrins (6, 7). Because β_2 integrins are the only known receptors for fibrinogen in PMNs (24), it appears that the dependence on β_2 integrins extends to signaling for pyk2 phosphorylation.

Lysates of PMNs stimulated with TNF for various times were next immunoprecipitated with anti-phosphotyrosine antibody and immunoblotted with anti-pyk2. Tyrosine phosphorylation of pyk2 was detectable at 20 minutes, intense at 40 minutes, and still marked by 60 minutes, compatible with the time course of TNF-induced spreading (3) (Figure 2b).

Activation of pyk2 by TNF. PMNs were lysed with nondenaturing buffer after a 60-minute incubation with or without TNF. Anti-pyk2 immunoprecipitates were washed, incubated in kinase buffer in the presence of [γ-^{32}P]ATP, separated by SDS-PAGE, transferred to a PVDF membrane, and subjected to autoradiography (Figure 3). The 2 species to undergo the most prominent increase in incorporation of [γ-^{32}P]ATP had M_r's compatible with pyk2 and IgG heavy chain. Additional species undergoing TNF-triggered phosphorylation had apparent M_r's of ~150, 70, 45, and 34 kDa. Similar amounts of pyk2 were precipitated in the 2 samples (data not shown). These experiments showed that pyk2 becomes more enzymatically active when PMNs are treated with TNF and/or associates with an active kinase in response to TNF.

TNF-induced association of pyk2 with paxillin. A major substrate to become tyrosine- and serine- or threonine-phosphorylated in PMNs in response to TNF is the focal adhesion protein paxillin (6). Paxillin can bind FAK (25) or pyk2 (26–30) by an interaction that is phosphotyrosine independent (25). In the previous experiment,(Figure 3), a protein with an M_r of ~70 kDa was immunoprecipitated with pyk2 from lysates of TNF-treated PMNs and phosphorylated in vitro, consistent with the M_r of paxillin. We therefore immunoprecipitated PMNs lysates with anti-pyk2 antibody and immunoblotted with anti-paxillin mAb. A small amount of paxillin was associated with pyk2 in control PMNs; the amount increased markedly upon stimulation by TNF (Figure 4a). The high apparent M_r of pyk2-associated paxillin suggested that both in basal and stimulated conditions, this fraction of paxillin was heavily phosphorylated, as previously documented for about 5% of paxillin in total lysates of TNF-treated PMNs (6). Results were the same when lysates were immunoprecipitated with anti-paxillin and immunoblotted with anti-pyk2 (Figure 4b).

Screening of a panel of tyrosine kinase inhibitors. Next, we tested a series of tyrosine kinase inhibitors for their ability to prevent the TNF-induced respiratory burst while sparing the respiratory burst triggered by the phorbol ester PMA. In dose-response fashion, we individually tested 51 structurally diverse compounds (Table 1) known to inhibit a variety of tyrosine kinases. Fourteen failed to exert more than 50% suppression of either the TNF- or PMA-induced respiratory burst up to the highest concentration of compound tested (7.7 μM). Another 10 were inhibitory but blocked the responses to TNF and to PMA equally, suggesting that they affected a shared pathway, were toxic, or interfered with the assay. Four were inhibitory for TNF response at higher concentrations than for PMA. Twenty-three inhibited the TNF response by more than 50% at or below 8 μM, and either did not inhibit the response to PMA or did so at a substantially higher concentration than required to inhibit the response to TNF. The 9 most selective compounds from the last group (i.e., most potent against the response triggered by TNF and least potent against the response triggered by PMA)

were further tested by adding them to PMNs 30 minutes in advance of TNF or PMA, which improved the apparent potency of several. Of these, tyrphostin A9 was the compound with the lowest IC_{50} for TNF (40 nM) and the greatest ratio between the IC_{50} for PMA and that for TNF (106-fold) (Figure 5a). Lack of toxicity of tyrphostin A9 and selectivity of its actions were illustrated not only by its sparing of the response to PMA over the concentration range 30 nM–2.6 μM, but also by the lack of effect on the ability of PMNs to ingest and kill *Salmonella typhimurium* (Figure 5b).

Inhibition of pyk2 phosphorylation by tyrphostin A9. A concentration of tyrphostin A9 that totally suppressed the TNF-induced respiratory burst while sparing the PMA-induced respiratory burst decreased the overall level of protein tyrosine phosphorylation stimulated by TNF in adherent cells. TNF-induced tyrosine phosphorylation of pyk2 was completely suppressed (Figure 5c).

Inhibition of pyk2 phosphorylation by wortmannin and PP2. The most potent inhibitor of the TNF-induced respiratory burst identified in previous work was wortmannin (31), an antagonist of PI3K. We wanted to determine what relation wortmannin's actions might have to the pathway involving pyk2. When added before or together with TNF, wortmannin inhibited H_2O_2 production with an IC_{50} of 0.8 ± 0.1 nM (mean ± SEM), whereas PMA-induced H_2O_2 release was unaffected (data not shown), demonstrating wortmannin's lack of toxicity. Delayed addition of 10 nM wortmannin still resulted in complete inhibition of H_2O_2 production triggered by previously added TNF, provided that the drug was added during the lag period characteristic of the response to TNF (2). Once the lag period had expired, addition of wortmannin had markedly less or no effect on the ongoing secretion of H_2O_2 (Figure 6a). Thus, a wortmannin-sensitive process was essential for initiating the respiratory burst in response to TNF, but not for maintaining it.

Next, we tested the effect of wortmannin of pyk2 phosphorylation, comparing it with the most selective src-family tyrosine kinase inhibitor commercially available – PP2 (32). Wortmannin (10 nM) had little effect on the overall level of protein tyrosine phosphorylation stimulated by TNF in adherent cells. Only 2 M_r classes of polypeptides (~125 and ~95 kDa, respectively) seemed to be affected (Figure 6b, top). Strikingly, however, tyrosine phosphorylation of pyk2 was completely abolished (Figure 6, bottom). In contrast, PP2 at much higher concentrations (2.5 μM) blocked almost all detectable tyrosine phosphorylation, including that of pyk2 (Figure 6b). Similar amounts of pyk2 were precipitated in each case (data not shown). These findings suggest that pyk2 participates in the signaling cascade leading to the respiratory burst in TNF-treated, adherent PMNs and that PI3K activity is required upstream of pyk2 activation. These results are also consistent with a role for src-family tyrosine kinases. However, attempts at reciprocal coimmunoprecipitation did not reveal a detergent-resistant association between pyk2 and fgr, hck, or syk (data not shown).

Table 1
Tyrosine kinase inhibitors tested for their effect on PMA- and TNF-stimulated H_2O_2 production.

Group A
- Daidzein
- Hypericin
- Tubercidin 5′-monophosphate
- 5,7-Dihydroxy-3-(4-hydroxyphenyl)-8-methoxy-4H-1-benzopyran-4-one
- 5,7-Dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one
- (3,4,5-Trihydroxybenzylidene)malononitrile
- *N*-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride
- 1-(5-Isoquinolinylsulfonyl)homopiperazine dihydrochloride
- 1-(5-Isoquinolinylsulfonyl)-2-methyl-piperazine
- Phosphonic acid ([(acetyloxy)methoxy]-2-naphthalenylmethyl)-*bis*([acetyloxy]methyl) ester
- *N*-(2-aminoethyl)-*N*-hexyl-5-isoquinolinesulfonamide hydrochloride
- Pseudohypericin
- (*s*)-5-Isoquinolinesulfonicacid 4-[2-[(5-isoquinolinyl-sulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl]phenyl ester
- *N*(4),*N*(6)-bis(Phenyl)-2-methyl-5-nitro-4,5,6-pyrimidinetriamine

Group B
- bis-Tyrophostin
- (–)-(*s*)-*N*-(α-Methylbenzyl)-3,4-dihydroxybenzylidenecyanoacetamide
- 3,5-Di-*tert*-butyl-4-hydroxybenzilidenecyanoacetamide
- *N*-Phenyl-3,4-dihydroxybenzylidencyanoacetamide
- 3-Amino-2,4-dicyano-5-(3′,4′,5′-trihydroxyphenyl)penta-2,4-dienonitrile
- *N,N*-Bis(2′-hydroxybenzyl)-3-aminosalicylic acid
- α-Cyano-3,4-dihydroxycinnamamide
- 3,4-Dihydroxy-α-cyanothiocinnamide
- 10-(3-[1-Piperazinyl]propyl)-2-trifluoromethylphenochiazine dimaleate
- Methyl 2,5-dihydroxycinnamate

Group C
- (4-Methoxybenzylidene)malononitrile
- *N*-(2′,5′-Dihydroxybenzyl)-*N*-[2′-hydroxybenzyl)-3-aminosalicylic acid
- *N*-(2′,5′-Dihydroxybenzyl)-5-aminosalicylic acid
- 3-[1-(3-Dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)-maleimide

Group D
- *N*-Benzyl-3,4-dihydroxy-—cyanocinnamide
- Methyl 2,5-dihydroxycinnamate
- (4-Hydroxybenzyl)malononitrile
- (6,7-Dimethoxyquinazolin-4-yl)-(3-bromophenyl)amine
- *N*-[2-(4-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride
- Chelerythrine chloride
- (+/–)1-(5-Isoquinolinesulfonyl)-3-methylpiperazine dihydrochloride
- *N*-(2′-Guanidinoethyl)-5-isoquinolinesulfonamide dihydrochloride
- *N*-(2-Aminoethyl)-5-chloro-1-naphthalenesulfonamide hydrochloride
- 1-(5-Isoquinolinylsulfonyl)piperazine hydrochloride
- *N*-(2-Aminoethyl)-5-isoquinolinesulfonamide dihydrochloride
- 1-(5-Chloronaphthalene-1-sulfonyl)-1h-hexahydro-1,4-diazepine hydrochloride
- 1-(5-Iodonaphthalene-1-sulfonyl)-1h-hexahydro-1,4-diazepine hydrochloride
- (*s*)-5-Isoquinolinesulfonicacid 4-[2-[(5-isoquinolinyl-sulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl]phenyl ester
- 4′-Amino-6-hydroxyflavone
- 2′,4′,6′-Trihydroxy-3-(4-hydroxyphenyl)propiophenone
- *N*-(2,3-Dihydroxyindol)-3,4-dihydroxybenzylidenecyanoacetamide
- *N*-(Ethylbenzyl)-3,4-dihydroxybenzylidenecyanoacetamide
- *N*-(Butylbenzyl)-3,4-dihydroxybenzylidenecyanoacetamide
- *N*-(3-Phenylpropyl)-3,4-dihydroxy-α-cyanocinnamide
- (–)-(*r*)-*n*-(α-Methylbenzyl)-3,4-dihydroxybenzylidenecyanoacetamide
- 3,4-Dihydroxybenzylidene)malononitrile
- 3,5-bis/*tert*-Butyl/-4-hydroxybenzylidene malononitrile

Compounds tested for their effect on PMA- and TNF-stimulated H_2O_2 production were subdivided into 5 groups as follows. Group A showed less than 50% inhibition of responses to both PMA and TNF at the highest tested concentration (7.7 μM). Group B showed 50% inhibition of responses to both PMA and TNF at approximately equivalent concentrations. Group C showed 50% inhibition of the response to PMA at concentrations lower than those causing 50% inhibition of the response to TNF. Group D showed 50% inhibition of the response to TNF at concentrations significantly lower than those causing 50% inhibition of the response to PMA.

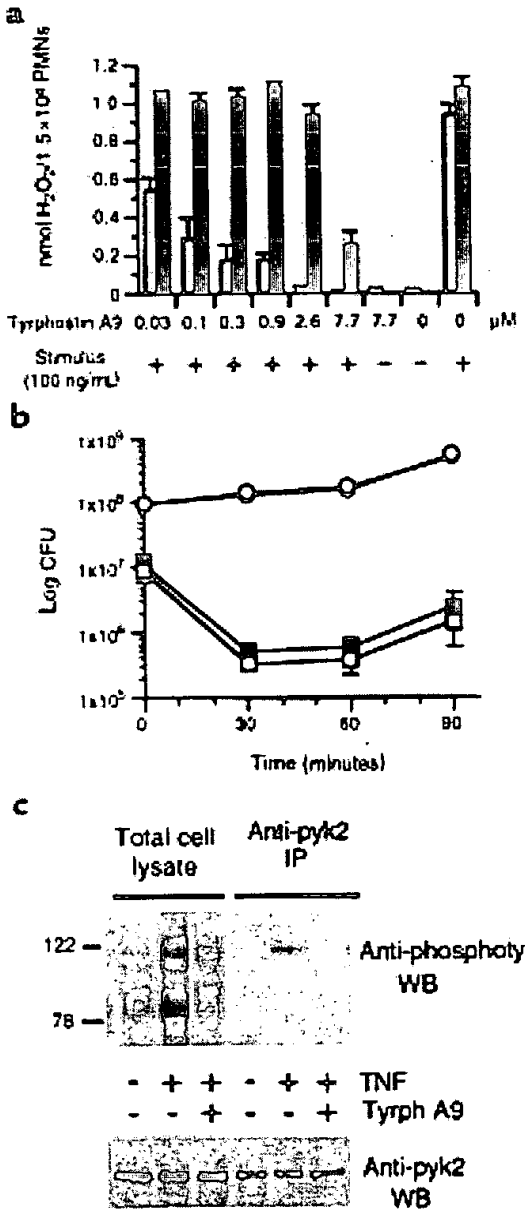


Figure 5

Tyrphostin A9 inhibits the TNF-induced respiratory burst of human PMNs and TNF-induced tyrosine phosphorylation of pyk2 but not their bactericidal activity. (a) Inhibition of TNF-stimulated H_2O_2 release. PMNs were plated in FBS-coated 96-well plates and preincubated for 30 minutes with the indicated concentrations of tyrphostin A9. Cells were then unstimulated (−) or stimulated (+) either with TNF (100 ng/mL; gray bars) or PMA (100 ng/mL; filled bars), and H_2O_2 release was recorded 60 minutes later as mean ± SEM of triplicates. (b) Lack of inhibition of bacterial killing. PMNs were preincubated (30 minutes at 4°C) with 0 (filled squares) or 5 μM (open squares) tyrphostin A9 and then infected with 10% autologous serum-opsonized *Salmonella typhimurium*. Cells were lysed at indicated times and CFUs were determined. Survival of bacteria without PMNs is shown with 0 (filled circles) or 5 μM (open circles) tyrphostin A9. Mean ± SEM of triplicates; most error bars fall within the symbols. (c) PMNs were plated on FBS-coated plates, preincubated for 30 minutes in the presence or absence of tyrphostin A9 (2 μM), and then stimulated with TNF (250 ng/mL) or left untreated for 60 minutes, as indicated. Cell lysates were immunoprecipitated (IP) with anti-pyk2 antibody. Total cell lysate and immunoprecipitates were separated by reducing SDS-PAGE, transferred to nitrocellulose, and Western blotted (WB) with anti-phosphotyrosine mAb (top) or anti-pyk2 antibody (bottom) followed by ECL detection. Molecular mass markers are indicated in kilodaltons.

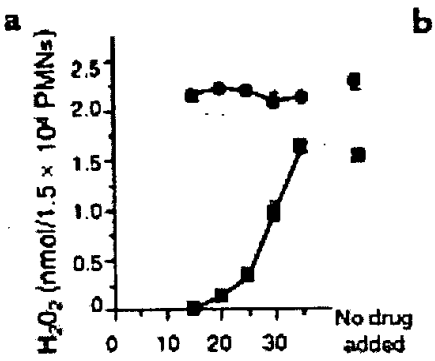
Inhibition of neutrophil spreading by wortmannin and tyrphostin A9. Both wortmannin and tyrphostin A9 inhibited TNF-induced spreading of adherent PMNs, but in a different fashion (Figure 7). Cells pretreated with wortmannin (10 nM) stayed mostly round after TNF treatment, extending only 1 or 2 short pseudopodia. In contrast, tyrphostin A9-treated cells responded to TNF by extending filopodia several micrometers long and began to flatten. Nonetheless, their spreading was arrested compared with TNF-stimulated cells treated with vehicle alone. Thus, wortmannin and tyrphostin A9 both interfered with TNF-induced cytoskeletal reorganization, but wortmannin appeared to act earlier in the sequence than tyrphostin A9.

Discussion

The present experiments demonstrate that pyk2 is present in primary human PMNs and that TNF triggers its tyrosine phosphorylation and activation in adherent cells; they also suggest that pyk2 plays an essential role in the TNF-triggered respiratory burst. The delayed and prolonged kinetic of pyk2's tyrosine phosphorylation is consistent with a downstream role in TNF signaling, such as that leading to cytoskeletal reorganization. The localization of pyk2 primarily to podosomes and focal adhesions positions it for this role, and its TNF-induced association with paxillin may be one manifestation of its execution of this assignment.

Adhesion-controlled cell responsiveness to soluble agonists has become a major paradigm in cell biology and immunology to explain how matrix and soluble signals combine to regulate cellular behavior and fate. In PMNs, adhesion-dependent responses that are independent of transcription and translation constitute an important portion of what the cells contribute to the survival of the organism as well as to its pathology. Experiments with blocking antibodies and/or genetic deficiency states have indicated that β_2 and β_3 integrins play a major role in the adhesion-dependent responses of PMNs (1, 33). It remains unknown, however, how integrins signal in this setting and how their signals are integrated with those elicited by soluble agonists. Both PI3K action and protein tyrosine phosphorylation are essential for physiological agonists to trigger secretion from adherent human PMNs (5, 9, 31, 34). Pyk2 is the only specific tyrosine kinase in PMNs shown to depend on PI3K for its activation. Thus, a key action of PI3K may be to engineer the activation of pyk2.

The observation that low-dose wortmannin interfered with the initial stages of TNF-induced cell spreading and blocked the TNF-induced tyrosine phosphorylation of pyk2 suggested that PI3K acted early in the TNF signal-



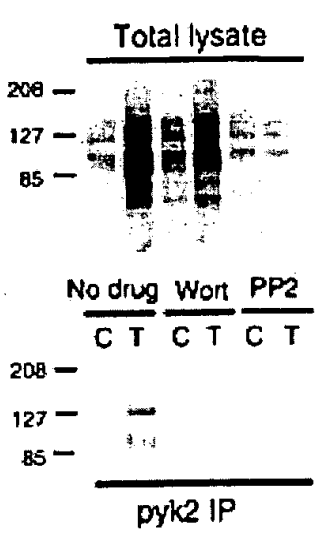


Figure 6
Wortmannin inhibits the TNF-induced respiratory burst and tyrosine phosphorylation of pyk2. (**a**) H_2O_2 release from PMNs measured 120 minutes after the addition of TNF (100 ng/mL; squares) or PMA (100 ng/mL; circles). Wortmannin (10 nM) was added to separate sets of cells at the indicated number of minutes after TNF or PMA. Results are mean ± SEM for triplicates in 1 experiment of 3; some error bars fall within the symbols. (**b**) Tyrosine phosphorylation. PMNs were plated on FBS-coated plates and treated for 60 minutes with buffer alone or TNF (100 ng/mL) in the presence or absence of wortmannin (Wort) at 10 nM or the src kinase inhibitor PP2 (2.5 μM) as indicated. Cell lysates were immunoprecipitated (IP) with anti-pyk2 antibody. Total cell lysate (top) and anti-pyk2 immunoprecipitates (bottom) were separated by reducing SDS-PAGE, transferred to nitrocellulose, and Western blotted (WB) with anti-phosphotyrosine mAb followed by ECL detection. Molecular mass markers are indicated in kilodaltons.

ing pathway and upstream of pyk2. However, wortmannin also blocked the TNF-induced respiratory burst when added at the end of the lag period at a time when cells were fully spread. This suggested that PI3K activity was also required after cell spreading had advanced past the relatively late point at which tyrphostin A9 could interrupt it. The latter observation suggests an additional role for PI3K downstream of pyk2.

Soluble, inflammatory stimuli of PMNs increase the affinity and number of β_2 integrins expressed at the cell surface. This process is thought to be mediated in large part by activation of PI3K (35). This could account for the action of PI3K upstream of pyk2, insofar as integrin activation is required to activate pyk2. PIP_2 and PIP_3 produced by PI3K can activate the serine protein kinase Akt (36). Akt can phosphorylate and activate protein kinase C and the phox47 component of the respiratory burst oxidase (37). Syk associates with both CD18 and fgr in activated, adherent PMNs (38), and activation of syk in response to formylated peptide appears to be required for cell spreading and paxillin phosphorylation (9). Binding of pyk2 to phosphorylated paxillin could help localize pyk2 to podosomes and focal adhesions. We speculate that activated syk and/or src kinases phosphorylate pyk2, and that phosphorylated pyk2 provides binding sites in podosomes and focal adhesions for additional molecules of PI3K. The ensuing tyrosine phosphorylation of PI3K would lead to its activation, membrane association, and further augmentation of integrin activation. This scenario could help explain why the long lag period in the TNF-stimulated respiratory burst has features of a positive-feedback system, terminating abruptly with the release of H_2O_2 at the maximal rate.

Defining the role of an enzyme within primary human neutrophils is more difficult than in many other types of cells. Neutrophils are not amenable to transfection. They cannot be loaded with presumptive dominant-negative peptides by osmotic shock or electroporation if the goal is to follow the cells' response to physiological agonists over subsequent hours. Leukemic cell lines such as HL-60 can respond to PMA

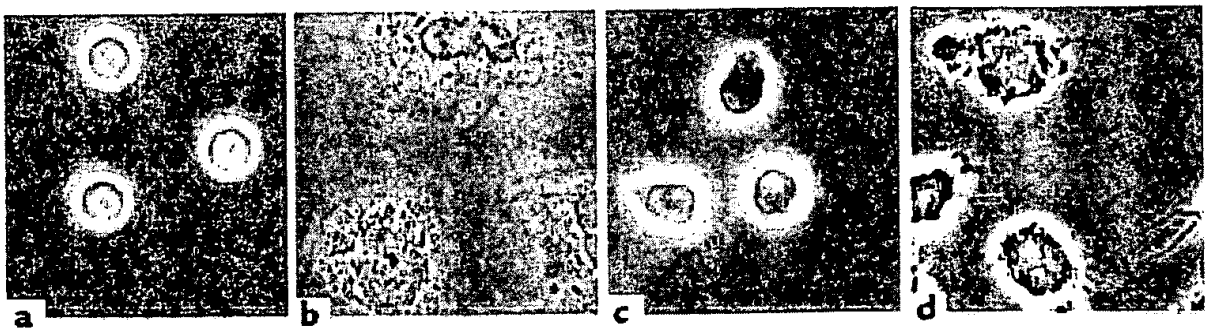


Figure 7
Wortmannin and tyrphostin A9 inhibit TNF-induced neutrophil spreading. PMNs adherent to FBS-coated glass coverslips were left untreated (**a**) or stimulated with TNF (100 ng/mL) for 60 minutes (**b–d**), either alone (**b**) or in the presence of wortmannin (10 nM) (**c**) or tyrphostin A9 (2 μM) (**d**), and then fixed and photographed with phase-contrast microscopy.

but do not manifest a TNF-stimulated respiratory burst after differentiation. Primary mouse neutrophils are genetically manipulatable through the germ line, but their TNF-induced respiratory burst is about 20-fold smaller than in human neutrophils (11) and, in our laboratory, has been detectable in only a small proportion of experiments; responses to a formylated peptide have been completely lacking (data not shown).

As an alternative to genetic intervention, we took a pharmacologic approach, surveying 51 tyrosine kinase inhibitors. We sought compounds at least equal in potency to those already shown to be inhibitory of the integrin-dependent, TNF-induced respiratory burst (6) and inactive against the response triggered by phorbol ester, which is integrin independent (1). Tyrphostin A9 was previously identified as a relatively selective inhibitor of the tyrosine kinase activity of the PDGF receptor, with an IC_{50} (40 nM) similar to that recorded in the present experiments (39). The potency of tyrphostin A9 on PMNs matches that of K252a, the most potent tyrosine kinase inhibitor of the TNF-induced respiratory burst yet described (IC_{50} = 30 nM), and exceeds by far the potency of others previously studied (genistein, IC_{50} = 10 μM; ST638, IC_{50} = 30 μM; piceatannol, IC_{50} = 1-10 μM) (5, 9). Tyrphostin A9's inhibition was relatively selective, sparing not only the PMA-induced respiratory burst but also the ability of the PMNs to ingest and kill bacteria. This illustrates the potential to exert an anti-inflammatory action without blocking intracellular bactericidal function.

Identification of the steps proximal and distal to pyk2 in the TNF- and adhesion-dependent responses of human PMNs may reveal relatively myeloid-specific, enzymatic targets for anti-inflammatory therapy.

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EX 2

Calcium Signaling by HBx Protein in Hepatitis B Virus DNA Replication

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Hepatitis B virus (HBV) infects more than 300 million people and is a leading cause of liver cancer and disease. The HBV HBx protein is essential for infection; HBx activation of Src is important for HBV DNA replication. In our study, HBx activated cytosolic calcium-dependent proline-rich tyrosine kinase–2 (Pyk2), a Src kinase activator. HBx activation of HBV DNA replication was blocked by inhibiting Pyk2 or calcium signaling mediated by mitochondrial calcium channels, which suggests that HBx targets mitochondrial calcium regulation. Reagents that increased cytosolic calcium substituted for HBx protein in HBV DNA replication. Thus, alteration of cytosolic calcium was a fundamental requirement for HBV replication and was mediated by HBx protein.

HBV is a para-retrovirus that replicates in the liver by reverse transcription but encapsidates a partially double-stranded 3-kb circular DNA genome (*1*). HBV replication requires the multifunctional HBx protein (*2*, *3*). In the cytoplasm, HBx stimulates Ras, Src, and c-Jun NH_2-terminal kinase (JNK) signal transduction pathways (*4–6*). HBx activation of Src promotes reverse transcription in the virus and DNA replication (*6–8*) as well as stimulation of several transcription factors (*1*). HBx might also interact in the nucleus with the transcriptional activator CREB/ATF (cAMP response element–binding protein/activating transcription factor) (*9–10*). HBx does not act on Src α-phosphatase or the COOH-terminal Src kinase (Csk) and does not bind Src kinases (*7*, *8*). Pyk2 is a cytoplasmic calcium–activated kinase that activates Src kinases (*11*) and downstream effectors, such as JNKs (*12*). Increased cytosolic calcium activates Pyk2, leading to its autophosphorylation at tyrosine amino acid position 402 (Y402), which creates a binding site for Src kinases and activates them. Because HBx activates the calcium-stimulated transcription factor NFAT (*13*), we investigated whether HBx acts on the calcium-Pyk2 pathway and whether calcium signaling is involved in stimulation of transcription and viral DNA replication.

Hepatic cell lines were transfected with vectors expressing HBx protein, a luciferase reporter dependent on transcription factor AP-1, and a dominant-inhibiting form of Pyk2 known as PKM (*14*). Inhibition of Pyk2 prevented HBx activation of AP-1 (*15*). Transfection of cells with one-eighth the amount of HBx expression plasmid reduced activation of AP-1 by only about one-half (*15*); these data indicated that PKM inhibits HBx activity rather than its expression. To determine whether HBx activates Pyk2, as indicated by Y402 phosphorylation (*14*), we transfected HepG2 cells with vectors expressing HBx or vector alone, with or without PKM expression. HBx induced increased phosphorylation of Pyk2 at Y402 by a factor of 4 without altering Pyk2 abundance (Fig. 1A), comparable to the effect of the phorbol ester tetradecanoyl phorbol acetate (*15*). HBx activation of Pyk2 was likely constitutive, as it was sustained for 48 hours after transfection, the last time point tested. The HBx-induced phosphorylation of Pyk2 correlated with stimulation of Pyk2 kinase activity (*15*).

To determine whether HBx activation of Pyk2 is essential for downstream stimulation of Src kinases, we transfected cells with vectors expressing HBx and PKM or with empty vector; Fyn was immunoprecipitated and tested for autophosphorylation activation by an in vitro kinase assay. HBx induced a factor of 5 increase in Fyn phosphorylation activity, which was prevented by coexpression with PKM (Fig. 1B). Thus, HBx activation of Pyk2 activated downstream Src kinases.

Studies were done to determine whether HBx acts on intracellular calcium to activate Pyk2. HBx-transfected cells showed increased phosphorylation of Pyk2 at Y402 by a factor of 5. Phosphorylation was inhibited by the cell-permeable cytosolic calcium chelator BAPTA-AM (Molecular Probes, Eugene, Oregon) at a concentration of 50 μM [twice the median inhibitory concentration (IC_{50})] (*15*). Thus, HBx activation of Pyk2 involves cytosolic calcium action. We therefore determined whether HBx acts on calcium channels in the endoplasmic reticulum, mitochondria, or plasma membrane for its activity. A low (0.5 mM) concentration of EGTA was added to the culture medium for 2 hours to block entrance of extracellular calcium (*16*), or cells were treated with BAPTA-AM (to block cytosolic calcium) or cyclosporin A (CsA). CsA predominantly binds mitochondrial cyclophilins, inhibits the mitochondrial transition pore, and disrupts mitochondrial calcium signaling (*17*, *18*). EGTA had no effect, whereas BAPTA-AM or CsA prevented HBx activation of Pyk2 (Fig. 1C), indicating that HBx likely acts on mitochondrial calcium control. A high concentration of EGTA (3 mM) did not block

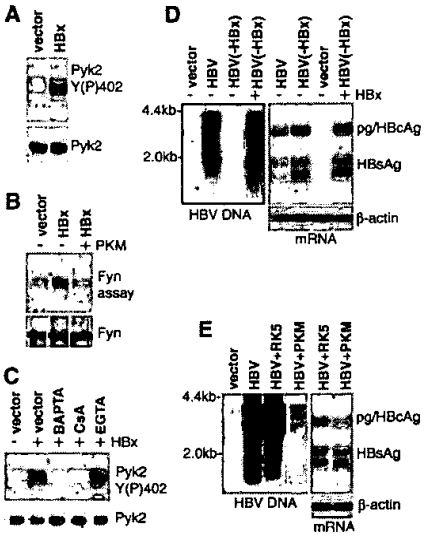

Fig. 1. (**A**) HepG2 cells were transfected with HBx expression plasmid or its empty plasmid (vector) (*7*, *8*, *29*) and with PKM plasmid that expressed a dominant-interfering Pyk2 or its empty plasmid (pRK5) (*14*). Cell lysates were resolved by gel electrophoresis, and immunoblot analyses were performed with antibodies to Pyk2 or Y(P)-402 Pyk2. (**B**) Fyn kinase was immunoprecipitated from cells and autophosphorylation activity (Fyn assay) determined in vitro using [γ-^{32}P]ATP, gel electrophoresis, and autoradiography (*7*, *8*). Fyn protein levels were determined by immunoblot. (**C**) HepG2 cells were treated for 2 hours with 50 μM BAPTA-AM, 3 μM CsA, or 0.5 mM EGTA and analyzed by immunoblot as above. (**D**) HepG2 cells were transfected with replication-competent wild-type genomic HBV DNA, an HBx(–) HBV genomic DNA, or vector, with or without an HBx expression plasmid (*15*). Cytoplasmic HBV core particles were isolated from equal numbers of cells, and viral DNA replication intermediates were detected by Southern blot hybridization (*8*). The smear represents 4-kb mature double-stranded to 2-kb single-stranded immature HBV DNA. Northern blot analysis was carried out using polyadenylated RNA extracted from equal numbers of cells. HBV mRNAs are indicated. (**E**) Southern and Northern blot analyses were performed on HepG2 cells transfected as above, with or without PKM or empty vectors. Quantification was performed by densitometry.

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TPA activation of Pyk2 phosphorylation but partially inhibited activation by HBx (*15*). It is likely that the effect of high levels of EGTA results from a partial requirement for low-level entry of calcium for full HBx-induced activation of Pyk2. Collectively, these data indicate that HBx alters cytosolic calcium regulation, probably by acting on mitochondria.

The requirement for HBx activation of Pyk2 in HBV replication was next examined. HepG2 cells were transfected with a 130% head-to-tail DNA copy of the HBV genome, which replicates in the livers of transgenic mice (*19*), in *Tupaia* hepatocytes in culture (*20*), and in an HBx-dependent manner in HepG2 cells (*21*). Expression of HBx from genomes was abolished by a targeted frameshift mutation (*21*). HepG2 cells were transfected with vector alone, wild-type HBV genomic DNA, or HBx(–) genomic DNA. Cytoplasmic viral core particles, the structures in which viral DNA replication takes place, were isolated, and the level of viral DNA replication was examined (Fig. 1D). HBV DNA replication was reduced by 95% in the absence of HBx expression, but was recovered by cotransfection of an HBx expression plasmid. Northern mRNA analysis showed no reduction in HBV pregenomic (pg)RNA and HBsAg mRNAs in the absence of HBx (Fig. 1D). Cotransfection of wild-type HBV genomic DNA with PKM reduced viral DNA replication by about 93%, similar to HBx(–) HBV samples, without altering viral mRNA levels (Fig. 1E). These results demonstrate that HBx specifically promotes HBV DNA replication in a Pyk2-dependent manner.

The requirement for cytosolic calcium in HBx-dependent viral replication was examined. Cells transfected with either wild-type or HBx(–) HBV genomic DNA were treated for 4 days with BAPTA-AM, CsA, or CGP37157, an inhibitor of the mitochondrial sodium-calcium pump (*17*). CGP37157 was used to provide an additional independent line of evidence for the importance of mitochondrial calcium in HBx-mediated HBV replication. Inhibitors were used at low levels, with no toxicity evident during treatment. CsA, BAPTA-AM, and CGP37157 all inhibited HBV replication in cytoplasmic core particles by 90 to 93% compared to untreated controls (Fig. 2A), similar in magnitude to inhibition of Pyk2 or the absence of HBx expression. Northern mRNA analysis showed a 50% reduction in pgRNA and HBsAg mRNAs (Fig. 2A). Cytosolic core particles were purified and incubated with [α-^{32}P]deoxynucleotide triphosphates (dNTPs) to examine endogenous HBV polymerase activity (Fig. 2B). In untreated controls, full-length double-stranded DNA products were produced, indicative of pgRNA reverse transcription and DNA-dependent DNA synthesis. PKM inhibition of Pyk2 or treatment of cells with CsA reduced DNA replication by 87 and 92%, respectively. In cells transfected with HBV genomic DNA and treated with low levels of BAPTA-AM for 4 days, viral DNA replication was reduced by 90%, whereas HBV mRNA levels decreased by less than half (Fig. 2C). These data show that HBx activation of HBV reverse transcription and DNA replication involves alteration of cytosolic calcium and coupled activation of Pyk2. The requirement for cytosolic calcium in HBx transcriptional stimulation was investigated in HepG2 cells transfected with luciferase reporters controlled by transcription factor AP-1 or CREB, with or without CsA treatment of cells (Fig. 2D). HBx activation of AP-1–dependent transcription was reduced almost 70% by treatment of cells with CsA (10 μg/ml). HBx stimulation of CREB-dependent transcription was resistant to high-dose CsA treatment, consistent with HBx activation of CREB by direct interaction (*22*). These data indicate that HBx transcriptional activation of AP-1, but not of CREB, requires alteration of cytosolic calcium.

We next investigated whether HBx activity can be replaced by reagents that increase cytosolic calcium. Cells were transfected with plasmids expressing wild-type HBV or HBx(–) DNA genomes and treated for 4 days with low, nontoxic levels of the calcium mobilizing agents valinomycin (1 nM) or thapsigargin (20 nM). Reverse transcription and DNA replication of (HBx)HBV was increased by valinomycin treatment by a factor of 20, to the same level as seen in wild-type (HBx+) virus, and by thapsigargin treatment by a factor of 10 (Fig. 3A). Neither agent increased cytoplasmic levels of HBV pgRNA or HBsAg mRNA. The low levels of thapsigargin or valinomycin induced a stimulation of Pyk2 activity by a factor of 3 or 5, respectively (Fig. 3B). These results demonstrate that HBx activation of viral reverse transcription and DNA replication can be replaced by agents that mobilize cytosolic calcium.

HBx acts on cytosolic stored calcium to stimulate Pyk2–Src kinase signal transduction pathways that activate HBV reverse transcription and DNA replication, and in some instances it functions as a moderate transcriptional activator. Three lines of evidence indicate that HBx stimulation of HBV reverse transcription and DNA replication involves alteration of cytosolic calcium and activation of Pyk2–Src kinase signal transduction: (i)

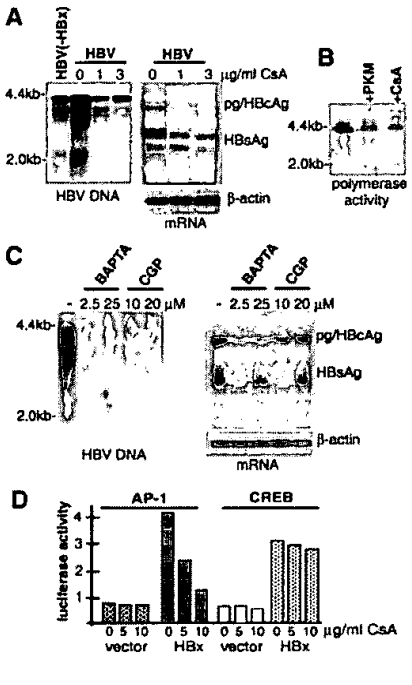

Fig. 2. HepG2 cells were transfected and treated for 4 days with CsA (**A** and **B**), BAPTA-AM, or CGP37157 (CGP) (**C**). Cytoplasmic HBV core particles were isolated, HBV DNA replication detected by Southern blot hybridization, and mRNA levels determined by Northern blot hybridization. In (**B**), endogenous polymerase activity of HBV pol protein was assayed in isolated cytoplasmic core particles obtained from equal numbers of cells using [α-^{32}P]dNTPs in vitro (*30*). Products were resolved by gel electrophoresis and autoradiography. (**D**) Cells were transfected with empty vector or HBx expression plasmid and luciferase reporters containing four binding sites for AP-1 or CREB, linked to a TATA box promoter (*15*). Cells were treated with CsA, as above, at the indicated dose and assayed for luciferase activity. A typical result is shown, which did not vary by more than 10% in three independent trials.

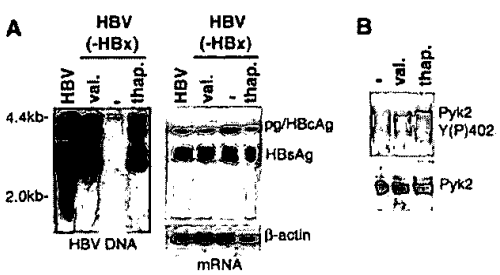

Fig. 3. (**A**) HepG2 cells were transfected with HBV or HBx(–) HBV genomic DNA, then treated with valinomycin (val.) or thapsigargin (thap.) for 4 days. Cytoplasmic core particles were isolated from equal numbers of cells, and HBV replication was examined by Southern blot hybridization. HBV transcription was analyzed by Northern blot hybridization. (**B**) Nontransfected HepG2 cells were treated with valinomycin or thapsigargin, and the abundance of Pyk2 or activated Pyk2 Y402 phosphorylation was determined by immunoblot.

Activation of Pyk2, which is critical for stimulation of HBV DNA replication in tissue culture, is typically mediated by increased levels of cytosolic calcium. Chelation of cytosolic calcium with BAPTA-AM blocked HBx activation of Pyk2 and HBV DNA replication. (ii) Inhibition of mitochondrial channels with CGP37157 or CsA blocked HBx activation of HBV DNA replication. (iii) Reagents that increase the level of cytoplasmic calcium functionally replace HBx in viral DNA replication. Thus, HBx acts on stored cytosolic calcium as a fundamental activity for HBV replication.

The molecular target for HBx-mediated calcium stimulation of HBV replication is not known. However, calcium stimulates a variety of protein kinases, and phosphorylation of the COOH-terminus of the HBV core protein is essential for viral replication (*1*), which suggests a possible link between the two. The regulation of intracellular calcium is central to the control of cellular metabolism, cell cycle, signal transduction, protein synthesis, transcription, and apoptosis (*18*). It is therefore not surprising that a number of viruses, including retroviruses such as HIV, rotovirus, adenovirus, and Rubella virus, encode regulatory proteins that alter normal calcium homeostasis to benefit one or more aspects of viral replication (*23–26*). A potential interaction between HBx and mitochondria has been reported (*27–29*), as well as a possibly modest dissolution of the mitochondrial transition pore potential (*29*). Taken collectively with this work, the alteration of cytosolic calcium is an important and central activity of HBV HBx protein and provides a new target for antiviral intervention.

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THROMBOSIS RESEARCH

REGULAR ARTICLE

Suppression of Protein Kinase C Is Associated with Inhibition of PYK2 Tyrosine Phosphorylation and Enhancement of PYK2 Interaction with Src in Thrombin-Activated Platelets

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Abstract

Blood platelets have recently been shown to express PYK2, a nonreceptor tyrosine kinase belonging to the FAK gene family. In this study, we examined the involvement of protein kinase C (PKC) in PYK2-related responses in human platelets. While PYK2 tyrosine phosphorylation induced by thrombin was inhibited by preincubation of platelets with PKC inhibitors, staurosporine and Ro31-8220, PYK2 association with Src was markedly enhanced under the same conditions. Platelet intracellular Ca^{2+} mobilization induced by thrombin was hardly inhibited by these PKC inhibitors. $p130^{Cas}$ is a docking protein that associates with FAK or PYK2 through the SH3 domain. Although we identified $p130^{Cas}$ in platelets for the first time, this docking protein failed to interact with PYK2. These results suggest that PKC activation (but not Ca^{2+} mobilization) is involved in PYK2 tyrosine phosphorylation and that PYK2 associates with Src without PYK2 tyrosine phosphorylation or $p130^{Cas}$ involvement in platelets.

Key Words: Platelets; PYK2; Src; Protein kinase C; Crk-associated substrate; Protein-tyrosine phosphorylation

It has been established that protein-tyrosine phosphorylation plays an important role in the induction of cellular functions, especially those related to cell growth and oncogenesis. Although platelets are terminally differentiated, nondividing cells, they possess high levels of tyrosine kinase activity [1,2]. The majority of tyrosine kinases in platelets reported to date are nonreceptor types, including Src family tyrosine kinases, Syk, and FAK [2]. The importance of tyrosine phosphorylation in platelet activation, especially that mediated by collagen receptor(s), Fcγ receptor II, and glycoprotein Ib/IX is now clear [3–7]. For example, collagen stimulation of platelets results in Syk activation, leading to phospholipase C-γ2 activation and the resultant intracellular Ca^{2+} mobilization [3,4].

Recently, a new nonreceptor protein-tyrosine kinase, PYK2 (also known as RAFTK and CAKβ), was identified [8–10]. The cDNA of PYK2 has closest homology (48% identity, 65% similarity) to FAK [9]. Both PYK2 and FAK lack SH2 and SH3 domains but, in their C-terminal region, possess two proline-rich domains that interact with the SH3

Abbreviations: PYK2, proline-rich tyrosine kinase 2; FAK, focal adhesion kinase; PKC, protein kinase C; $p130^{Cas}$, Crk-associated substrate; MAPK, mitogen-activated protein kinase; MoAb, monoclonal antibody.

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domain of $p130^{Cas}$ and $p105^{HEF1}$ [8,11]. PYK2 was shown to be phosphorylated through integrin-mediated pathways, intracellular Ca^{2+} mobilization, and protein kinase C (PKC) activation in several cells, including human B cells, CMK cells, and PC12 cells [8,12–14]. PYK2 is involved in mitogen-activated protein kinase (MAPK) activation, which is initiated by G protein-coupled receptors and dependent on Ca^{2+} [15,16]. It was also reported that PYK2 associates with the SH2 domain of Src family tyrosine kinases via its phosphotyrosine [13,17,18], which is involved in Ras/MAPK cascade [18].

PYK2 is abundantly expressed in both megakaryocytes and platelets, in addition to brain tissue [9]. Furthermore, the phosphorylation of PYK2 reportedly is modulated by integrin interaction with fibronectin or collagen in CMK megakaryocytic cells [13]. Accordingly, it can be assumed that PYK2 is involved in the signaling pathways of platelets, in which signals resulting from integrin engagement are related to important functional responses. In fact, Raja et al. recently revealed that tyrosine phosphorylation of platelet PYK2 is dependent on PKC and actin polymerization and does not require integrin-mediated platelet aggregation [19]. However, not much is known about the mechanism by which PYK2 is activated or its interaction with other tyrosine kinases in platelets. In this study, we studied the involvement of PKC in PYK2-related responses and found that PYK2 interaction with Src was markedly enhanced by PKC inhibition in thrombin-stimulated platelets.

1. Materials and Methods

1.1. Materials

Ro31-8220, a specific PKC inhibitor [20,21], was a gift from Rosche Products (Welwyn Garden City, Herts, UK). The following materials were obtained from indicated suppliers: thrombin (Green Cross, Osaka, Japan); anti-PYK2 polyclonal antibody, anti-Src monoclonal antibody (MoAb), and anti-phosphotyrosine MoAb (4G10) (Upstate Biotechnology, Lake Placid, NY); anti-PYK2 MoAb, anti-$p130^{Cas}$ MoAb, and anti-phosphotyrosine MoAb (PY20) (Transduction Laboratories, Lexington, KY); anti-Syk MoAb and leupeptin (Wako Pure Chemical Industries, Tokyo, Japan); control mouse IgG (Zymed Laboratories, San Francisco, CA); bovine serum albumin (BSA) and Na_3VO_4 (Sigma Chemical Co., St. Louis, MO); protein A–Sepharose 4B and protein G–Sepharose (Pharmacia Biotech, Uppsala, Sweden); staurosporine (Kyowa Medex, Tokyo, Japan); fura-2 AM (Dojindo Laboratories, Kumamoto, Japan); peroxidase-conjugated goat anti-mouse IgG (Cappel, Durham, NC); ECL chemiluminescence reaction reagents (Amersham, Buckinghamshire, UK); [^{32}P] orthophosphate (8500-9120 Ci/mmol) (Du Pont-New England Nuclear, MA).

1.2. Platelet Preparation

Platelet-rich plasma was prepared as previously described (22). The platelets were washed and resuspended in a buffer containing 138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1 mg/ml of glucose, and 20 mM HEPES (pH 7.4). Just before centrifugation of platelet suspensions, a 15% volume of acid-citrate-dextrose A solution and 0.1 μM prostaglandin I_2 were added to inhibit platelet activation. The final platelet suspensions were adjusted to 1.0×10^9 cells/ml unless otherwise stated. When intracellular Ca^{2+} concentrations were measured, 100 μM EGTA was supplemented.

1.3. Immunoprecipitation and Western Blotting

Platelets were stimulated at 37°C as indicated and reactions were terminated with an equal volume of 2×ice-cold lysis buffer (2% Triton X-100, 100 mM Tris/HCl (pH 7.2), 2 mM EGTA, 2 mM Na_3VO_4, 1 mM PMSF, and 100 μg/ml of leupeptin). All subsequent steps of immunoprecipitation were carried out at 4°C. After extensive sonication, the lysates were centrifuged at 15000×*g* for 5 minutes and then precleared with protein A–Sepharose 4B or protein G–Sepharose. The resultant supernatants were incubated for 2 hours with the antibody indicated. Protein A–Sepharose or protein G–Sepharose was then added and further incubated for 1 hour. The immune complexes were washed with 1×lysis buffer three times and then resuspended in SDS-PAGE sample buffer. The proteins were resolved on 8% SDS-PAGE and then electrophoretically transferred to PVDF membrane. The membranes were blocked with 1% BSA in phosphate-buffered saline (PBS). After extensive wash-

ing with PBS containing 0.1% Tween-80, the immunoblots were incubated with anti-PYK2 MoAb (0.25 μg/ml), antiphosphotyrosine MoAb (1 μg/ml of PY20 plus 1 μg/ml of 4G10), anti-Src MoAb (0.16 μg/ml), or anti-p130^{Cas} MoAb (0.25 μg/ml) for 2 hours. Antibody binding was detected using peroxidase-conjugated goat anti-mouse IgG and visualized with ECL chemiluminescence reaction reagents. When indicated, tyrosine phosphorylation of PYK2 was quantified using PDI420oe scanner and Quantity one 2.5a software for Macintosh.

1.4. Measurement of Intracellular Ca^{2+} Concentration ($[Ca^{2+}]i$)

$[Ca^{2+}]i$ measurement was performed with the use of Ca^{2+}-sensitive fluorophore fura-2 as described previously [23]. The platelet count was adjusted to 2×10^8 cells/ml in a buffer containing 100 μM EGTA. The $[Ca^{2+}]i$ values were determined from the ratio of fura-2 fluorescence intensity at 340 and 380 nm excitation.

1.5. Platelet PKC Activation

PKC activation in intact platelets was evaluated by pleckstrin (p47) phosphorylation as described previously [24].

2. Results and Discussion

We first investigated PYK2 tyrosine phosphorylation in thrombin-stimulated platelets by immunoprecipitation with anti-PYK2 polyclonal antibody and Western blotting with antiphosphotyrosine antibody. As reported previously [19], PYK2 underwent rapid tyrosine phosphorylation, with a maximum level obtained as early as 2 minutes after stimulation with thrombin (Figure 1). Thrombin is a potent physiologic agonist and stimulates most of the signaling pathways in platelets; for example, Ca^{2+} mobilization, PKC activation, protein-tyrosine phosphorylation, and Ras/MAPK cascade [25,26]. Among these pathways, Ca^{2+} mobilization and PKC activation (after phosphoinositide hydrolysis) are most important factors in thrombin-stimulated platelets [25]. It is likely that PYK2 phosphorylation in platelets challenged with thrombin

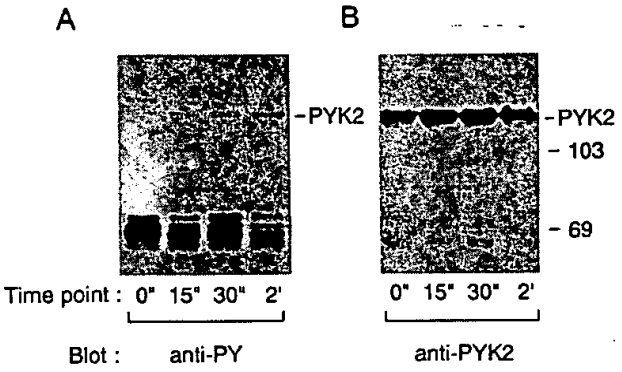

Fig. 1. Tyrosine phosphorylation of PYK2 in thrombin-stimulated platelets. Human platelets were challenged with 1 U/ml of thrombin for various durations, as indicated. The platelet protein lysates were immunoprecipitated with anti-PYK2 polyclonal antibody, resolved on 8% SDS-PAGE, and then immunoblotted with anti-phosphotyrosine MoAb (A) or anti-PYK2 MoAb (B). The data are representative of four experiments.

is secondary to one or more of these signaling pathways.

We then analyzed the involvement of PKC in PYK2-related responses. We first examined the effects of commonly used PKC inhibitors, Ro31-8220 and staurosporine, on PYK2 tyrosine phosphorylation induced by thrombin. Staurosporine is a powerful but nonspecific protein kinase inhibitor [27,28], while Ro31-8220 is a weaker but specific PKC inhibitor [20,21]. Under the conditions used, thrombin-induced PKC activation in intact platelets, as evaluated by pleckstrin phosphorylation, was abolished by preincubation of the cells with Ro31-8220 (10 μM) or staurosporine (1 μM; data not shown). The treatment of platelets with these PKC inhibitors resulted in marked inhibition of thrombin-elicited PYK2 tyrosine phosphorylation (Figure 2A). We also examined tyrosine phosphorylation of Syk, which is a nonreceptor tyrosine kinase expressed in hematopoietic cells and plays an important role in eliciting platelet functional responses [2,3,6,7]. While staurosporine abolished Syk tyrosine phosphorylation induced by thrombin, Ro31-8220 did not (data not shown). These results suggest that Ro31-8220 acts as a specific PKC inhibitor in platelets and that its inhibition of PYK2 tyrosine phosphorylation is related to its PKC inhibition but not to its nonspecific effects. These findings are also consistent with the general idea that staurosporine, unlike Ro31-8220, is a non-

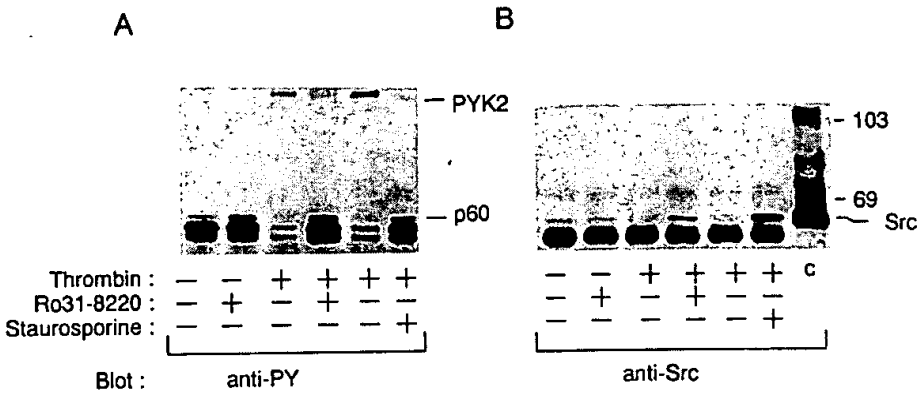

Fig. 2. Effects of Ro31-8220 or staurosporine on PYK2 tyrosine phosphorylation (A) and PYK2 interaction with Src (B) in platelets stimulated with thrombin. Platelets pretreated without or with 10 μM Ro31-8220 or 1 μM staurosporine for 5 minutes were stimulated with 1 U/ml of thrombin for 2 minutes. Immunoprecipitates obtained with anti-PYK2 polyclonal antibody were resolved on 8% SDS-PAGE and probed with anti-phosphotyrosine MoAb (A) or anti-Src MoAb (B). The data are representative of three experiments.

specific protein kinase inhibitor [27,28]. Although PKC involvement in PYK2 tyrosine phosphorylation has already been described in several cells, including platelets [8,14,19], pathways that are independent of PKC have also been proposed. For example, intracellular Ca^{2+} mobilization is reported to play a crucial role in PYK2 phosphorylation [8,16]. Hence, we compared platelet intracellular Ca^{2+} mobilization with the level of PYK2 tyrosine phosphorylation. Under the conditions where Ro31-8220 and staurosporine inhibited thrombin-induced PYK2 phosphorylation (Figure 3C), these inhibitors had only a marginal effect on platelet peak $[Ca^{2+}]i$ changes but markedly suppressed the decay of Ca^{2+} signals induced by thrombin (Figures 3A and 3B). Hence, we suggest that Ca^{2+} mobilization does not play an important role in inducing PYK2 tyrosine phosphorylation at least in platelets. Although PYK2 tyrosine phosphorylation induced by the Ca^{2+} ionophore A23187 (1 μM) was reported in platelets [19], this effect cannot be ascribed solely to Ca^{2+} mobilization; high concentrations of Ca^{2+} ionophore alone can activate several platelet signaling pathways in addition to Ca^{2+} mobilization, including PKC activation [29].

Furthermore, we noticed a 60-kDa tyrosine-phosphorylated protein coimmunoprecipitated with PYK2, which was most evident in platelets preincubated with PKC inhibitors and stimulated with thrombin (Figure 2A). Since platelets abundantly express Src, the molecular weight of which is 60 kDa [1,2], we performed Western blotting with anti-Src MoAb to see whether this tyrosine-phosphorylated protein is Src. As expected, the 60-kDa protein was Src; Src weakly associated with PYK2 in resting platelets and dissociated from PYK2 upon thrombin activation (Figure 2B). When platelets were preincubated with PKC inhibitors, Ro31-8220 and staurosporine, before thrombin challenge, the Src-PYK2 interaction was markedly augmented. Ro31-8220 or staurosporine alone failed to induce this interaction. Under the same conditions, these PKC inhibitors abolished thrombin-induced PYK2 tyrosine phosphorylation, as described above (Figure 2A). The major phosphorylation site of PYK2, Tyr-402, reportedly serves as a binding site for the SH2 domain of Src family tyrosine kinases [13,17,18]. However, it is unlikely that the phosphotyrosine residue of PYK2 associates with the SH2 domain of Src in our experiments, since the association between PYK2 and Src was enhanced despite lowered tyrosine phosphorylation of PYK2. Alternatively, direct interaction between Src tyrosine phosphorylation site(s) and PYK2 can be postulated but is not likely because PYK2, like FAK, does not possess the SH2 domain or other phosphotyrosine binding motif.

We were thus led to postulate the presence of other factors that mediate indirect interaction between PYK2 and Src. Various proteins have been reported to mediate FAK-Src interaction [30] and such proteins may be responsible for the PYK2-

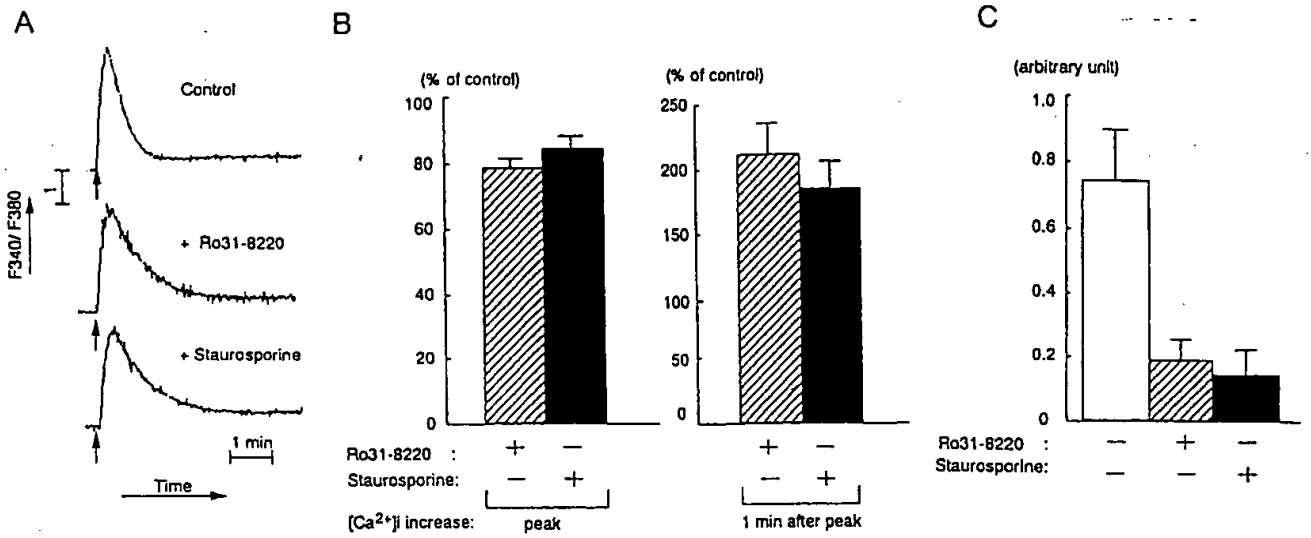

Fig. 3. Effects of Ro31-8220 or staurosporine on platelet intracellular Ca^{2+} mobilization (A, B) and PYK2 tyrosine phosphorylation (C) induced by thrombin. (A) Platelets pretreated without (top) or with 10 μM Ro31-8220 (middle) or 1 μM staurosporine (bottom) for 5 minutes were stimulated with 1 U/ml of thrombin, and the $[Ca^{2+}]i$ changes were monitored by the ratio of fura-2 fluorescence (340/380 nm). (B) Effects of Ro31-8220 or staurosporine on thrombin-induced intracellular Ca^{2+} mobilization were evaluated at the time points where thrombin induced peak $[Ca^{2+}]i$ increases and 1 minute passed after the peak formation. The results were expressed as a percentage of the control (parallel thrombin stimulation without pretreatment). Data represent the mean±SD (n=3). The fact that both Ro31-8220 and staurosporine markedly augment the thrombin-induced $[Ca^{2+}]i$ increases 1 minute after the peak formation indicates that these PKC inhibitors suppress the decay of thrombin-elicited intracellular Ca^{2+} mobilization. (C) The levels of PYK2 tyrosine phosphorylation were observed under the same conditions as in (A) and quantified as described in Materials and Methods. Data represent the mean±SD (n=3).

Src interaction. One of the most eligible candidates is $p130^{Cas}$, a recently-identified docking protein which belongs to a new family of structurally related proteins (Cas-related proteins) [30]. This protein may act as a docking protein not only with FAK but also PYK2 through its SH3 domain [11, 30–32]. As shown in Figure 4, $p130^{Cas}$ was clearly detected in the immunoprecipitates with anti-$p130^{Cas}$ MoAb and in the whole platelet lysate. To our knowledge, this is the first report to demonstrate its presence in platelets. We further examined the involvement of $p130^{Cas}$ in the PYK2-Src interaction. When immunoprecipitates with anti-PYK2 antibody were resolved on SDS-PAGE and then immunoblotted with anti-$p130^{Cas}$ MoAb, $p130^{Cas}$ was not detected in the immunoprecipitates (Figure 5). This was also true even under the conditions where maximal PYK2-Src interaction was induced by pretreatment with PKC inhibitors and stimulation with thrombin (Figure 5; see also Figure 2). The failure of this interaction was also confirmed by Western blotting with anti-PYK2 MoAb

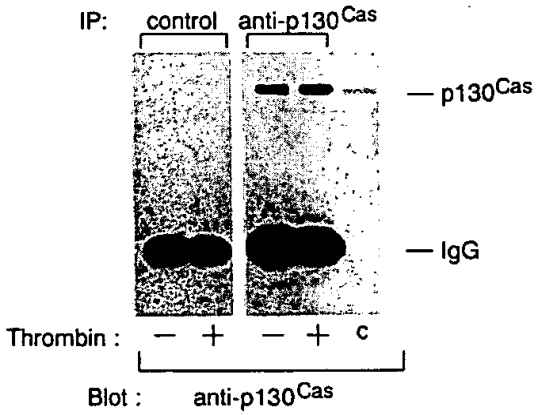

Fig. 4. Identification of $p130^{Cas}$ in human platelets. Platelets were stimulated without or with 1 U/ml of thrombin for 2 minutes. Immunoprecipitates obtained with control-mouse IgG or anti-$p130^{Cas}$ MoAb, along with whole platelet lysates (c), were resolved on 8% SDS-PAGE and then immunoblotted with anti-$p130^{Cas}$ MoAb. The data are representative of three experiments.

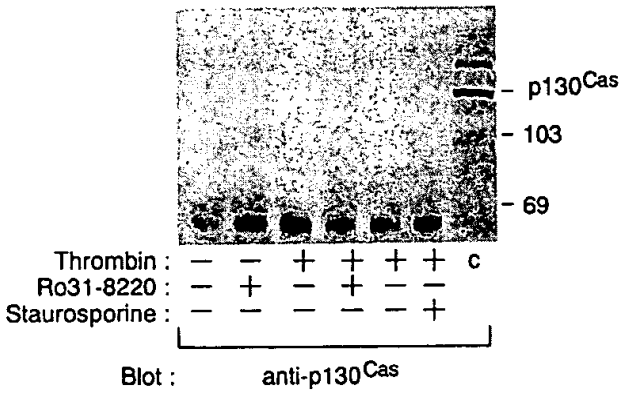

Fig. 5. Failure of PYK2 to interact with $p130^{Cas}$. Platelets were pretreated without or with 10 μM Ro31-8220 or 1 μM staurosporine for 5 minutes and then stimulated with 1 U/ml of thrombin for 2 minutes. Immunoprecipitates obtained with anti-PYK2 polyclonal antibody, along with whole platelet lysates (c), were resolved on 8% SDS-PAGE and probed with anti-$p130^{Cas}$ MoAb. The data are representative of three experiments.

of immunoprecipitates with anti-$p130^{Cas}$ MoAb (data not shown). Although indirect interaction between Src and PYK2 is strongly suggested in platelets, it is unlikely that $p130^{Cas}$ is involved in this interaction.

Staurosporine is a potent, nonselective protein kinase inhibitor [27,28]. In fact, staurosporine inhibits phosphorylation of serine/threonine and tyrosine residues of platelet proteins, including that of PYK2, FAK, Syk, PKC, and myosin light chain kinase (data not shown). Ro31-8220, which selectively inhibits PKC [20,21], inhibited the phosphorylation of PKC and PYK2, but not Syk, as described above. Under the conditions where the interaction between PYK2 and Src was augmented by staurosporine or Ro31-8220, PYK2 phosphorylation was abolished but the decay of thrombin-induced intracellular Ca^{2+} mobilization was markedly suppressed (Figure 3A). Accordingly, it is possible that intracellular Ca^{2+} plays an important role in the PYK2-Src interaction. Clarification of the precise molecular mechanisms by which PYK2 becomes associated with Src will be important for further investigation.

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An Intracellular Calcium Signal Activates p70 but Not p90 Ribosomal S6 Kinase in Liver Epithelial Cells*

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In the rat liver epithelial cell lines GN4 and WB, angiotensin II (Ang II) activates the G_q class of regulatory G-proteins, increasing intracellular calcium, protein kinase C activity, and protein tyrosine phosphorylation. We compared the ability of Ang II and other compounds that increase intracellular calcium (*i.e.* the calcium ionophore A23187 and thapsigargin) or protein kinase C activity (the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate) to activate p70 ribosomal S6 kinase ($p70^{S6K}$) and p90 ribosomal S6 kinase ($p90^{RSK}$). In GN4 cells, increasing intracellular calcium stimulated $p70^{S6K}$ activity in a rapamycin- and wortmannin- sensitive manner, but did not affect $p90^{RSK}$ activity. In contrast, 12-*O*-tetradecanoylphorbol-13-acetate strongly activated $p90^{RSK}$ but only weakly stimulated $p70^{S6K}$. The ability of calcium to activate $p70^{S6K}$ was confirmed by blocking the A23187-dependent activation through chelation of extracellular calcium with EGTA; the effect of thapsigargin was inhibited by the cell permeant chelator bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM). Similarly, BAPTA-AM prevented the activation of $p70^{S6K}$ by Ang II, suggesting that this signal was largely calcium-dependent. In contrast, the Ang II-dependent activation of mitogen-activated protein kinase and $p90^{RSK}$ was not inhibited but was enhanced by BAPTA-AM. These results show that in GN4 cells, Ang II selectively activates $p70^{S6K}$ through effects on calcium, $p90^{RSK}$ through effects on protein kinase C. The activation of $p70^{S6K}$ by calcium stimuli or Ang II was independent of calmodulin but correlated well with the activation of the recently identified, nonreceptor calcium-dependent tyrosine kinase (CADTK)/PYK-2. Both calcium- and Ang II-dependent activation of $p70^{S6K}$ were attenuated by the tyrosine kinase inhibitor genistein, and activation of $p70^{S6K}$ was higher in GN4 than WB cells, correlating with the increased expression and activation of CADTK/PYK-2 in GN4 cells. In summary, these results demonstrate that intracellular calcium selectively activates $p70^{S6K}$ in GN4 cells, consistent with increased CADTK/PYK-2 signaling in these cells.

The peptide hormone angiotensin II (Ang II)[1] initiates multiple intracellular processes that in some cell types influence cell proliferation. The seven-transmembrane-spanning Ang II receptor is coupled to the G_q class of regulatory G-proteins, which, when activated, stimulates inositol phosphate-mediated calcium release, protein kinase C activity, and additional signals regulated by $\beta\gamma$ subunits (1). In rat liver epithelial cells, an additional response to Ang II is a rapid (15–60 s), calcium-dependent increase in tyrosine phosphorylation of proteins (2, 3). One of the tyrosine-phosphorylated proteins has been purified from Ang II-treated rat liver epithelial cells using ATP affinity chromatography as the penultimate step and shown to be the major calcium-dependent protein tyrosine kinase (CADTK) in these cells (4). Although not directly activated by calcium *in vitro*, this kinase is stimulated by treatment of cells with agonists and hormones that elevate intracellular calcium (3, 4). Peptide sequencing of the purified kinase and cDNA cloning using rat liver epithelial cell mRNA revealed a novel cytoplasmic tyrosine kinase highly related to the p125 focal adhesion kinase (5). The kinase has also been recently identified by three other groups. One group using a polymerase chain reaction strategy to search for novel kinases cloned the human sequence PYK-2 and have also shown its activation in a calcium- and PKC-dependent manner (6). Two other groups screening for kinases related to p125 focal adhesion kinase have reported the sequence as cell adhesion kinase β and related adhesion focal tyrosine kinase (7, 8).

In addition to stimulation of calcium and calmodulin, PKC, MAPK, c-Jun N-terminal protein kinase (JNK), and CADTK/PYK-2 (1, 9, 10), Ang II has been shown to activate the ribosomal p70 S6 kinase ($p70^{S6K}$) (11). This ubiquitous class of mitogen-activated protein kinases is best known for their ability to phosphorylate the S6 protein of the 40 S ribosome (reviewed in Ref. 12). Although related in sequence to another S6 kinase ($p90^{RSK}$), the regulation of $p70^{S6K}$ is clearly distinct from the $p90^{RSK}$, the latter being activated as part of the Ras-dependent MAPK cascade (13). In contrast to $p90^{RSK}$, activation of the $p70^{S6K}$ pathway is inhibited by rapamycin (14, 15). In mammals, the intracellular target for rapamycin is the binding protein FKB12, which forms a complex with the recently identified protein kinases known as FRAP and RAFT (16, 17). Similar in sequence homology to the phosphatidylinositol-3

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[1] The abbreviations used are: Ang II, angiotensin II; MAPK, mitogen-activated protein kinase; BAPTA-AM, bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetraacetoxymethyl ester; CADTK, calcium-dependent tyrosine kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PI-3 kinase, phosphatidylinositol-3 kinase; EGF, epidermal growth factor; TBST, Tris-buffered saline/Tween; PBS, phosphate-buffered saline; PKC, protein kinase C; $p70^{S6K}$, p70 ribosomal S6 kinase; $p90^{RSK}$, p90 ribosomal S6 kinase; JNK, c-Jun kinase; PAGE, polyacrylamide gel electrophoresis; RIPA, radioimmunoprecipitation assay; RAFT, rapamycin and FKBP12 target; FRAP, FKBP-rapamycin-associated protein.

kinases (PI-3 kinases) (18), the FRAP/RAFT kinase is required for activation of $p70^{S6K}$ (16) but does not appear to phosphorylate phosphatidylinositol, PI-3 kinase, or $p70^{S6K}$. Thus the mechanism by which FRAP/RAFT regulates $p70^{S6K}$ signaling remains to be elucidated.

The $p70^{S6K}$ can be activated by numerous stimuli, including growth factors, inhibitors of protein synthesis (cycloheximide or anisomycin), and, as noted above, hormones such as Ang II (11, 19, 20). Complete activation of $p70^{S6K}$ appears to require multisite phosphorylation by several protein kinases, at least one of which is proline-directed and phosphorylates a sequence(s) in the C-terminal region of the S6 kinase (21–24). Despite the knowledge of specific phosphorylation sites, the activating "S6 kinase kinases" remain to be identified. In addition to the involvement of the rapamycin-sensitive FRAP/RAFT mentioned above, $p70^{S6K}$ is inhibited by wortmannin and LY294002, implicating PI-3 kinase in the signaling to $p70^{S6K}$ (25, 26). These results are further supported by studies involving expression of a constitutively active PI-3 kinase (27). A potential intermediary for PI-3 kinase in $p70^{S6K}$ activation is the serine and threonine kinase PKB, also known as Akt (28, 29), which has been shown to activate $p70^{S6K}$ when co-expressed in cells (30).

The $p70^{S6K}$ is involved in mitogenesis; studies with neutralizing antibodies to $p70^{S6K}$ or the inhibitor rapamycin have shown that this kinase is required for G_1 progression (31–33). The ability of $p70^{S6K}$ to phosphorylate the ribosomal S6 protein has been well characterized (34), but the contribution of this event to the regulation of protein synthesis is less clear (reviewed in Ref. 12). Phosphorylation of the S6 protein has been found to enhance the translation of mRNAs containing 5′-polypyrimidine tracts (35, 36), and it is likely that $p70^{S6K}$ plays a role in growth-related control of translation. For example, translation of specific messages such as those encoding elongation factor 1α (36) or insulin-like growth factor II (37) appear to be regulated by the S6 pathway; these may be members of a class of proteins required for progression through the G_1 phase of the cell cycle.

Ang II also stimulates protein synthesis in a number of cell types (11, 38), an event that may be dependent on calcium (39) and $p70^{S6K}$ (11). Because of our interest in calcium and Ang II signaling, we compared the ability of these stimuli to activate the $p70^{S6K}$ in liver epithelial cells, cells known to have a proliferative response to Ang II (1). In addition, Ang II leads to a protein kinase C-independent increase in AP1 binding, as well as stimulation of MAP kinase and c-Jun N-terminal kinase (10) and a calcium-dependent activation of a novel; calcium-dependent tyrosine kinase (CADTK). In this article we describe the finding that agents and hormones that increase intracellular calcium activate $p70^{S6K}$ but not $p90^{RSK}$. Furthermore, these studies suggest that the initial steps in Ang II action are well correlated with activation of CADTK.

EXPERIMENTAL PROCEDURES

Materials—Human Ang II (DRVYIHPF) was obtained from Sigma and prepared in 50 mM acetic acid prior to use. The S6 peptide (RRLSSLRA) (40) and PKI peptide (TTYADFIASGRTGRRNAIHD) (41) were synthesized by Dr. D. Klapper (University of North Carolina, Chapel Hill, NC). Thapsigargin, A23187, bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calyculin A, wortmannin, rapamycin, anisomycin, and calmidizolium were obtained from Biomol and prepared in Me_2SO. Calmodulin, leupeptin, and aprotinin were obtained from Sigma. Epidermal growth factor (EGF) was obtained and used as described previously (10). The intact 40 S ribosomal subunit was purified from rat liver by the procedure of Terao and Ogata (42).

Cell Culturing and Harvesting—Rat liver epithelial cells (GN4 or WB) were grown at 37 °C in Richter's minimal essential media containing 0.1 μM insulin and supplemented with 10% fetal bovine serum in a humidified 5% CO_2 atmosphere as described earlier (3). Cells were serum starved (0.1% fetal bovine serum) for 20–24 h prior to agonist stimulation. Cells were washed twice with phosphate-buffered saline (PBS) and once with Buffer H (50 mM β-glycerophosphate, pH 7.4, 1.5 mM EGTA, 0.15 mM sodium orthovanadate, 1 mM dithiothreitol, 25 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), after which they were scraped in 0.5 ml of Buffer H. The cells were then sonicated with two 5-s pulses (Fisher MDL 550 Sonic Dismembrator), and the lysates were centrifuged at 100,000 × *g* (4 °C).

$p70^{S6K}$ Assays—The activity of $p70^{S6K}$ in cell lysates was determined as described earlier (43). The 100,000 × *g* supernatants were assayed for $p70^{S6K}$ activity by monitoring the phosphorylation of the ribosomal protein S6 (2 μg) in a buffer (30 μl) containing 25 mM β-glycerophosphate (pH 7.4), 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 10 mM $MgCl_2$, 10 μM calmidizolium, 2 μM PKI peptide, and 100 μM [γ-^{32}P]ATP (2000 μCi/mmol) for 20 min at 30 °C. The reactions were terminated by the addition of SDS-PAGE sample buffer (30 μl), heated, and applied to SDS-PAGE (10% acrylamide). The radioactive band corresponding to the ribosomal S6 protein was identified by staining with Coomassie Blue and autoradiography; the radioactive band was excised from the dried gel and quantitated by liquid scintillation counting. Calmidizolium (10 μM) was included in the assay mixture to prevent the phosphorylation of the ribosomal S6 protein by calcium- and calmodulin-dependent kinases.

Immunoprecipitation Assays for $p70^{S6K}$ and $p90^{RSK}$—In some experiments, $p70^{S6K}$ was assayed by immunoprecipitation of the $p70^{S6K}$ after lysis of the cells in Tris/Triton lysis buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 nM calyculin A, and 148 μM Na_3VO_4). The samples were immunoprecipitated as described below and the immunoprecipitate/protein A-agarose beads were washed once with Tris/Triton lysis buffer and twice with PBS (1 ml). The S6 kinase assays were performed using a modification of the above assay procedure. In these assays, the immunoprecipitate/protein A-agarose beads (20 μL) were assayed in 20 mM Hepes, pH 7.3, 10 mM β-glycerophosphate, 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 50 μM [γ-^{32}P]ATP (1000 μCi/mmol) plus 250 μM S6 peptide in a final reaction volume of 60 μl. The assay was performed at 30 °C for 20 min with constant shaking in an Eppendorf 5436 Thermomixer. The reaction was terminated by the addition of 20 μl of 100 mM EDTA (pH 7.0); the samples were centrifuged for 5 min at 13,000 × *g*, and the supernatant (40 μl) was spotted on Whatman P-81 paper. The papers were washed in 10% phosphoric acid, and the radioactivity incorporated into S6 peptide was determined by liquid scintillation counting. The assays for $p90^{RSK}$ were performed exactly as described above, except the cell lysates were immunoprecipitated with a rabbit polyclonal antibody developed against the C terminus of $p90^{RSK}$ as described previously (Ref. 44; kindly provided by Dr. Edwin G. Krebs, Department of Pharmacology, University of Washington).

$p70^{S6K}$ Immunoprecipitation and Immunoblotting—For immunodetection of $p70^{S6K}$, cells were washed as described above and lysed in RIPA buffer containing 20 mM Tris (pH 7.4), 137 mM NaCl, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1.0% Triton X-100, and 2.0 mM EDTA plus 10 nM calyculin A and 0.5 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 13,000 × *g* for 10 min (4 °C). The samples were immunoprecipitated using a rabbit polyclonal antibody developed against a C-terminal peptide (QAFPMISKRPEHLRMNL) of the $p70^{S6K}$ (kindly provided by Dr. J. Weiel, Glaxo-Wellcome). Immunoprecipitation was facilitated by the addition of protein A-agarose (20 μl, packed beads), and the immunoprecipitates were washed three times with RIPA buffer prior to addition of SDS-PAGE sample buffer. Samples were applied to SDS-PAGE and blotted to polyvinylidene difluoride, and the immunoprecipitated $p70^{S6K}$ was detected by incubating blots with the C-terminal $p70^{S6K}$ antibody (diluted 1:2500 in TBST). The immunoblot was incubated with goat anti-rabbit (alkaline phosphatase-conjugated) antibodies (diluted 1:5000 in TBST), and the color was developed according to the manufacturer's procedure (Promega).

Measurement of CADTK Tyrosine Autophosphorylation—The activity of CADTK was assayed by determining the amount of tyrosine autophosphorylation by a modification of the procedure described earlier (4). Briefly, the cells were washed twice with PBS and harvested in RIPA or Tris/Triton lysis buffer as described above for the S6 kinase immunoblotting experiments. The CADTK was immunoprecipitated with a rabbit polyclonal antibody developed against a glutathione *S*-transferase-CADTK fusion protein (5). The CADTK immunoprecipitates were washed three times with RIPA or Tris/Triton lysis buffer and applied to SDS-PAGE (8% acrylamide). After electrophoresis the pro-

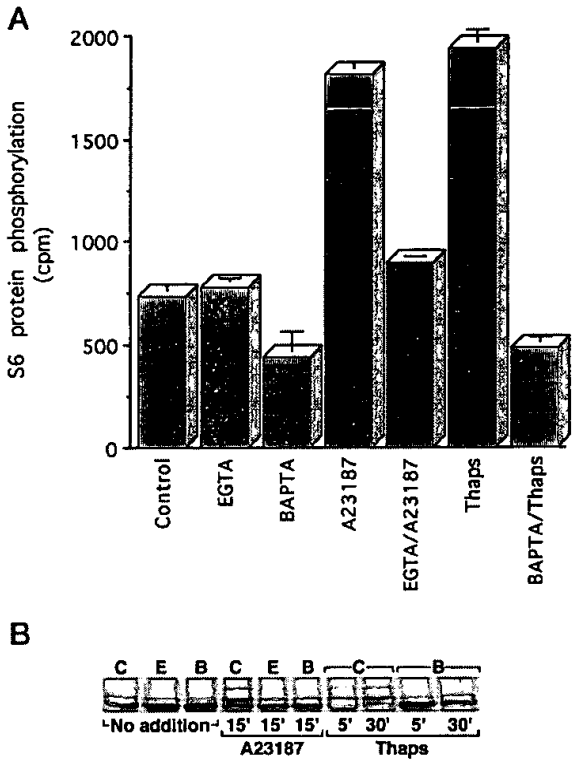

FIG. 1. **Calcium agonists activate p70^{S6K}.** *A*, GN4 cells were grown in 10-cm dishes and serum-starved as described under "Experimental Procedures." The cells were then incubated with 1 μM A23187 or 2 μM thapsigargin (*Thaps*) for 10 min. In some experiments, the calcium chelators EGTA (5 mM) and BAPTA (50 μM) were added to the medium 15 min prior to the addition of calcium stimuli. The cells were harvested and assayed for S6 kinase activity as described under "Experimental Procedures," and the S6 kinase activity is plotted as radioactivity (cpm, ^{32}P) incorporated into the ribosomal S6 protein. The results represent the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of n = 2 experiments. *B*, cells were prepared as described in *A* and treated with calcium agonists for the lengths of time indicated at the *bottom*. In some experiments cells were incubated with PBS (*C*), 5 mM EGTA (*E*), or 50 μM BAPTA (*B*) for 15 min prior to the addition of calcium stimuli. The cell lysates were prepared by washing the cells twice with PBS and adding 1 ml of RIPA buffer to solubilize the cells. The cell lysate was clarified by centrifugation at 12,000 × *g* prior to the addition of antisera to p70^{S6K}. The immunoprecipitated p70^{S6K} was identified by SDS-PAGE and immunoblotting as described under "Experimental Procedures."

teins were transferred electrophoretically to nitrocellulose, and the amount of phosphotyrosine on CADTK was quantitated by antiphosphotyrosine immunoblotting (PT66) and ECL as described earlier (4).

RESULTS

Calcium Activates p70^{S6K} but Not p90^{RSK}—Incubation of GN4 rat liver epithelial cells with compounds that increase intracellular calcium (*i.e.* the ionophore A23187, thapsigargin (which releases calcium from internal stores; Ref. 45), or Ang II), stimulates intracellular protein tyrosine phosphorylation (2). To investigate the influence of calcium-stimulated tyrosine phosphorylation on "downstream" kinase signaling pathways, we examined the ability of calcium to activate the ribosomal S6 kinase p70^{S6K}. Incubation of GN4 cells with 1 μM A23187 or 2 μM thapsigargin increased S6 kinase activity as measured by the phosphorylation of the 40 S ribosomal S6 protein (Fig. 1*A*). A typical response to these stimuli was a 2–3-fold increase over basal levels, similar to that produced by EGF (See Fig. 3). The calcium-stimulated increase in S6 kinase activity was inhibited by the calcium chelators EGTA and BAPTA-AM. Preincubation with EGTA prevented activation of p70^{S6K} by A23187; the cell-permeant chelator BAPTA-AM (BAPTA) inhibited both the thapsigargin- and ionophore-stimulated S6 kinase activity (Fig. 1*A*). These results indicate that for these agonists the increase in p70^{S6K} activity is calcium-dependent, and with thapsigargin, the effect is primarily dependent on intracellular calcium.

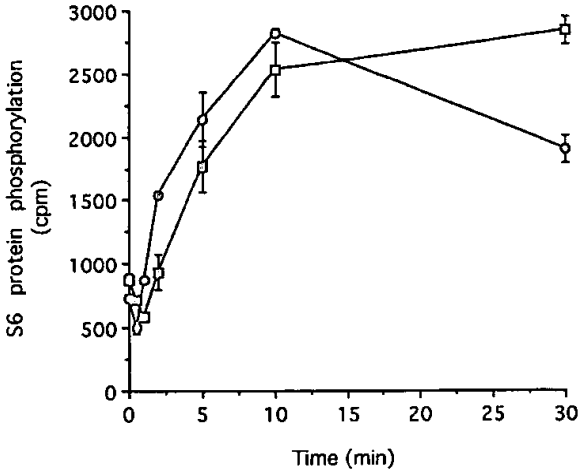

FIG. 2. **Angiotensin II and thapsigargin increase p70^{S6K} activity.** GN4 cells were prepared as described in the legend to Fig. 1 and incubated with 1 μM Ang II (○) or 2 μM thapsigargin (□) for the lengths of time indicated. The cells were harvested, and the cell lysates were assayed for S6 kinase activity as described earlier. The S6 kinase activity is plotted as radioactivity (cpm, ^{32}P) incorporated into the ribosomal S6 protein, and the results represent the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of n = 3 experiments.

In addition to the increased the phosphorylation of the ribosomal S6 protein, calcium activated p70^{S6K} as demonstrated by SDS-PAGE and immunoblotting analysis (Fig. 1*B*). In these experiments, p70^{S6K} was immunoprecipitated from cell lysates and immunoblotted for p70^{S6K} as described under "Experimental Procedures." Four immunoreactive bands corresponding to the multiple phosphorylated forms of p70^{S6K} were detected (46). In all experiments, increased p70^{S6K} activity (as determined by S6 protein phosphorylation) correlated with an upward mobility shift of both the slowest and fastest migrating immunoreactive bands on SDS-PAGE. The increased upward mobility shift of p70^{S6K} produced by A23187, or thapsigargin was prevented by preincubation with calcium chelators (Fig. 1*B*). BAPTA preincubation reduced the mobility shift of p70^{S6K} from untreated and A23187- or thapsigargin-treated cells, demonstrating that BAPTA affected both the stimulated and basal S6 kinase activity. In contrast, EGTA inhibited only the A23187-stimulated S6 kinase mobility shift, similar to the results obtained by assaying S6 protein phosphorylation. (Fig. 1, *A* and *B*).

Like thapsigargin, Ang II rapidly increases intracellular calcium and tyrosine phosphorylation in rat liver epithelial cells (2). As shown in Fig. 2, incubation of GN4 cells with Ang II rapidly stimulated p70^{S6K} activity comparable with that observed with thapsigargin. The increase in p70^{S6K} activity was apparent as early as 2–5 min after Ang II addition, with the peak of S6 phosphorylation occurring after 10–20 min. The effect of Ang II and thapsigargin was sustained for more than 60 min (data not shown). Similar results were found by analyzing immunoblots of p70^{S6K}; Ang II, thapsigargin, and A23187 stimulated a time-dependent increase in the p70^{S6K} mobility shift on SDS-PAGE, which paralleled the increase in S6 protein phosphorylation (see Fig. 4*A*).

In cardiac myocytes, Ang II and calcium have been reported to stimulate the activity of MAPK and the ribosomal S6 kinase p90^{RSK} (47). Although Ang II activated MAPK in GN4 cells by

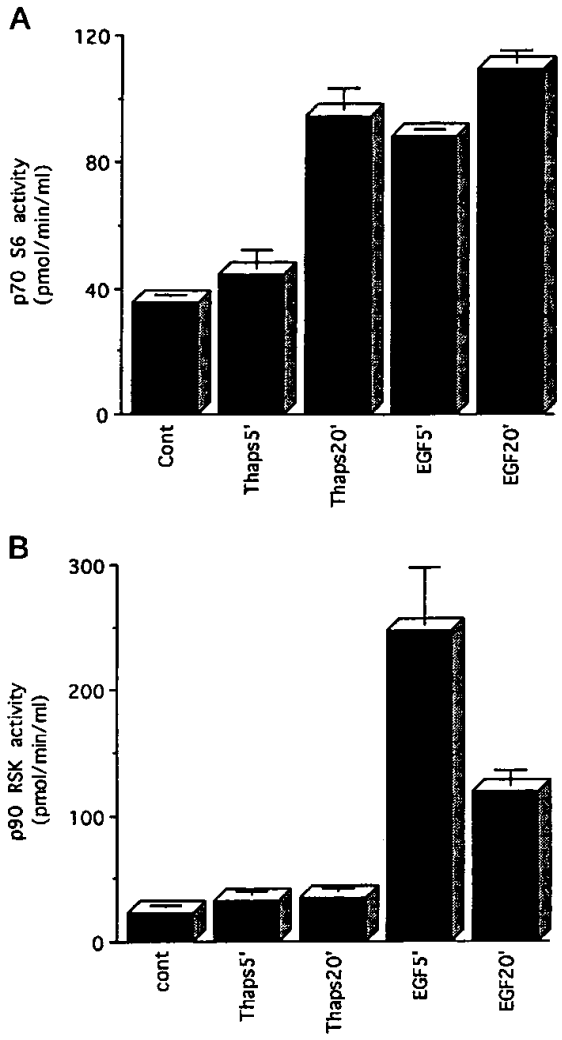

FIG. 3. **Thapsigargin activates p70^{S6K}, but not p90^{RSK}.** Serum-starved GN4 cells were incubated with 2 μM thapsigargin (*Thaps*) or 6.6 nM EGF for the lengths of time indicated. The cells were washed two times with PBS and harvested in Tris/Triton lysis buffer as described under "Experimental Procedures." The lysate from each 10-cm dish was immunoprecipitated with antibodies specific for p70^{S6K} (*A*) or p90^{RSK} (*B*) and assayed for S6 peptide kinase activity as described above. The S6 peptide activity is plotted as the amount of activity (pmol/min/ml), and the results represent the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 3$ experiments. *cont*, control.

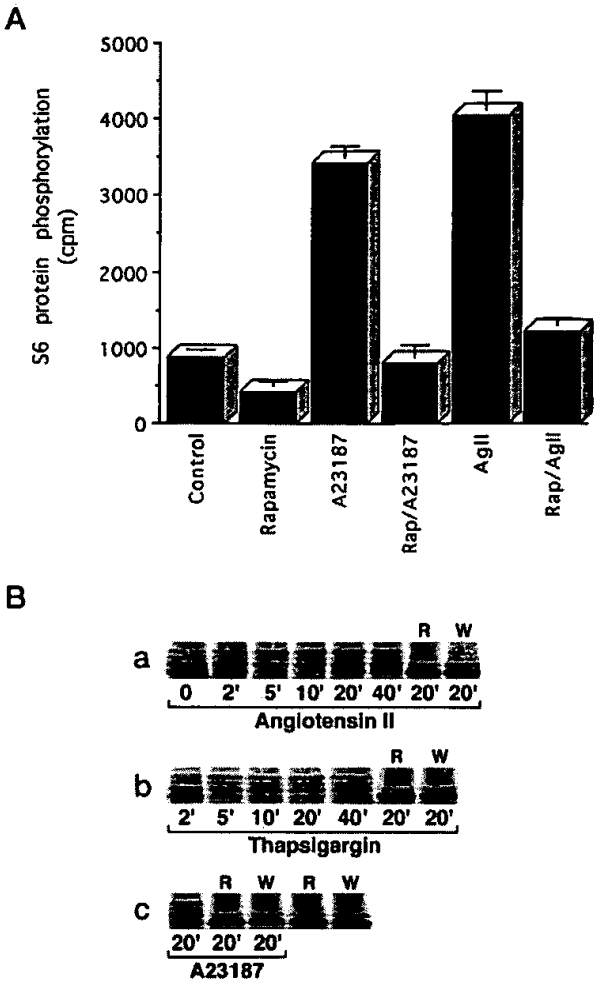

FIG. 4. **Rapamycin and wortmannin inhibit the activation of p70^{S6K} by calcium agonists.** *A*, GN4 cells were incubated with 0.1% Me_2SO (carrier for rapamycin) or rapamycin (*Rap*, 10 nM, 15 min) prior to the addition of A23187 (1 μM) or Ang II (*AgII*, 1 μM). Cell lysates were prepared as described earlier, and the S6 kinase activity is plotted as radioactivity (cpm, ^{32}P) incorporated into the ribosomal S6 protein. The results represent the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 3$ experiments. *B*, GN4 cells were incubated with 1 μM Ang II, 2 μM thapsigargin or 1 μM A23187 for the lengths of time indicated. In some experiments, cells were incubated with 10 nM rapamycin (*R*) or 50 nM wortmannin (*W*) for 15 min prior to the addition of agonist. The p70^{S6K} was immunoprecipitated and detected by immunoblotting as described in the legend to Fig. 1.

a PKC-dependent mechanism, thapsigargin does not stimulate MAPK in these cells (5, 10). We therefore compared the ability of thapsigargin and EGF to activate p70^{S6K} and p90^{RSK} in GN4 cells. As expected, both agonists stimulated p70^{S6K} (Fig. 3*A*), but only EGF stimulated p90^{RSK} in GN4 cells (Fig. 3*B*). The kinase assay on p90^{RSK} immunocomplexes using the S6 peptide (RRLSSLRA) as a substrate also demonstrated that A23187 did not activate p90^{RSK}, whereas Ang II potently activated this enzyme, as previously reported (10) (data not shown).

Calcium-dependent p70^{S6K} Activation Is Inhibited by Wortmannin and Rapamycin—To further rule out the possibility that p90^{RSK} was contributing to the phosphorylation of the S6 protein in cell lysates and to investigate the mechanism by which calcium stimulates p70^{S6K}, we examined the effect of compounds known to inhibit p70^{S6K} activation. Rapamycin has been previously shown to inhibit p70^{S6K} without affecting p90^{RSK} activity (14, 15). As shown in (Fig. 4*A*), incubation of cells with 10 nM rapamycin completely inhibited the Ang II- and calcium ionophore (A23187)-stimulated increase in p70^{S6K} S6 peptide kinase activity. Similarly, rapamycin blocked the thapsigargin-dependent activation of p70^{S6K}; (unstimulated, 35.7 ± 0.4 pmol/min/ml; thapsigargin, 94.3 ± 7.1 pmol/min/ml; thapsigargin and rapamycin, 23.9 ± 0.2 pmol/min/ml). Rapamycin also blocked Ang II-, A23187-, and thapsigargin-dependent p70^{S6K} activation, as assessed by the gel mobility immunoblotting (Fig. 4*B*). These results demonstrate that Ang II and calcium stimulate p70^{S6K} through a rapamycin-sensitive pathway and confirm that p70^{S6K} and not p90^{RSK} is the major ribosomal S6 kinase activated by calcium stimuli in GN4 cells.

Studies with the inhibitor wortmannin and constitutively active forms of PI-3 kinase have demonstrated a role for this enzyme in the regulation of p70^{S6K} (25–27). To determine whether calcium stimulated p70^{S6K} through a PI-3-kinase-dependent pathway, the effect of wortmannin on p70^{S6K} activity was examined. As shown in Fig. 4*B*, incubation of GN4 cells with wortmannin (50 nM) inhibited the mobility shift of p70^{S6K} stimulated by Ang II, A23187, or thapsigargin. The inhibition

of $p70^{S6K}$ by wortmannin occurred at low concentrations (10–50 nM), consistent with the effects of this compound on PI-3 kinase (48). Similar inhibitory affects of wortmannin were found on the calcium-dependent activation of S6 kinase, as determined by S6 protein phosphorylation (data not shown). Thus, this calcium-stimulated signaling pathway appears to be mediated through both a PI-3 kinase and FRAP/RAFT-dependent pathway.

Angiotensin II Activates $p70^{S6K}$ in a Calcium-dependent Manner—Ang II stimulates an inositol phosphate-mediated increase in intracellular calcium (reviewed in Ref. 49), and since our results demonstrated that calcium stimulates $p70^{S6K}$ activity, we examined whether the activation by Ang II was calcium-dependent. Incubation of GN4 cells with the calcium chelator BAPTA inhibited the Ang II-stimulated S6 kinase activity in a dose-dependent manner, with >90% inhibition occurring at 50 μM BAPTA (Fig. 5*A*). Like the results found with S6 kinase activity, BAPTA inhibited the Ang II-stimulated $p70^{S6K}$ mobility shift (Fig. 5*B*) at concentrations similar to those required to inhibit the activation by A23187 or thapsigargin (Fig. 1, *A* and *B*). Chelation of extracellular calcium with EGTA (5 mM) only minimally affected the Ang II-stimulated S6 kinase activity, further supporting the thesis that intracellular calcium was required for the activation of $p70^{S6K}$ (Fig. 5*B*). The calcium-dependent activation of $p70^{S6K}$ occurred independently of calmodulin, since the calmodulin inhibitor calmidizolium (50) did not inhibit this event. Similarly, neither calmidizolium nor W-7 prevented the autophosphorylation of CADTK, although calmidizolium (15 μM) inhibited the Ang II-dependent activation of the myosin light chain peptide kinase activity, a known calmodulin-dependent process (data not shown). In comparison, preincubation of GN4 cells with BAPTA did not inhibit but significantly stimulated the activation of $p90^{RSK}$ by Ang II in a dose-dependent manner (Fig. 5*C*). Furthermore, incubation with BAPTA alone (50 μM) increased the basal level of $p90^{RSK}$ to levels equivalent to that of Ang II stimulation in these cells. Similar to the results obtained with $p90^{RSK}$, BAPTA pretreatment potently enhanced the basal and Ang II-stimulated level of MAPK activity (data not shown).

PKC Activates $p90^{RSK}$ but Not $p70^{S6K}$ in GN4 Cells—Since Ang II increases diacylglycerol formation and PKC activity in GN4 cells, we investigated whether the activation of $p70^{S6K}$ by Ang II was also PKC-dependent. Incubation of cells with phorbol ester (TPA, 100 nM) for 20 min did not stimulate $p70^{S6K}$ activity in these cells, although $p90^{RSK}$ was stimulated by TPA (100 nM, 5 min) (Fig. 6, *A* and *B*). To further investigate the involvement of PKC, PKC activity was down-regulated by chronic incubation with TPA (1 μM, 24 h). The activation of $p70^{S6K}$ by Ang II was not inhibited by this treatment, indicating that PKC did not play a substantial role in regulating $p70^{S6K}$ in these cells (Fig. 6*A*). In comparison, this treatment effectively eliminated the activation of $p90^{RSK}$ by TPA as expected (Fig. 6*B*).

The Activation of $p70^{S6K}$ by Calcium Requires a Tyrosine Kinase—In epithelial cells, the Ang II- and calcium-stimulated increase in protein tyrosine phosphorylation is inhibited by the tyrosine kinase inhibitor genistein (3, 51). GN4 cells were briefly incubated with genistein (10–200 μM) to test the ability of this compound to prevent the activation of $p70^{S6K}$ by Ang II or calcium stimuli. As shown in Fig. 7*A*, genistein inhibited the Ang II-stimulated $p70^{S6K}$ activity in a dose-dependent manner, suggesting the involvement of a tyrosine kinase in the calcium and Ang II signaling to $p70^{S6K}$. Similarly, genistein inhibited the activation of $p70^{S6K}$ by thapsigargin (data not shown). Recently, we have purified the major calcium- and Ang II-stimulated tyrosine kinase (CADTK) from GN4 rat liver epi-

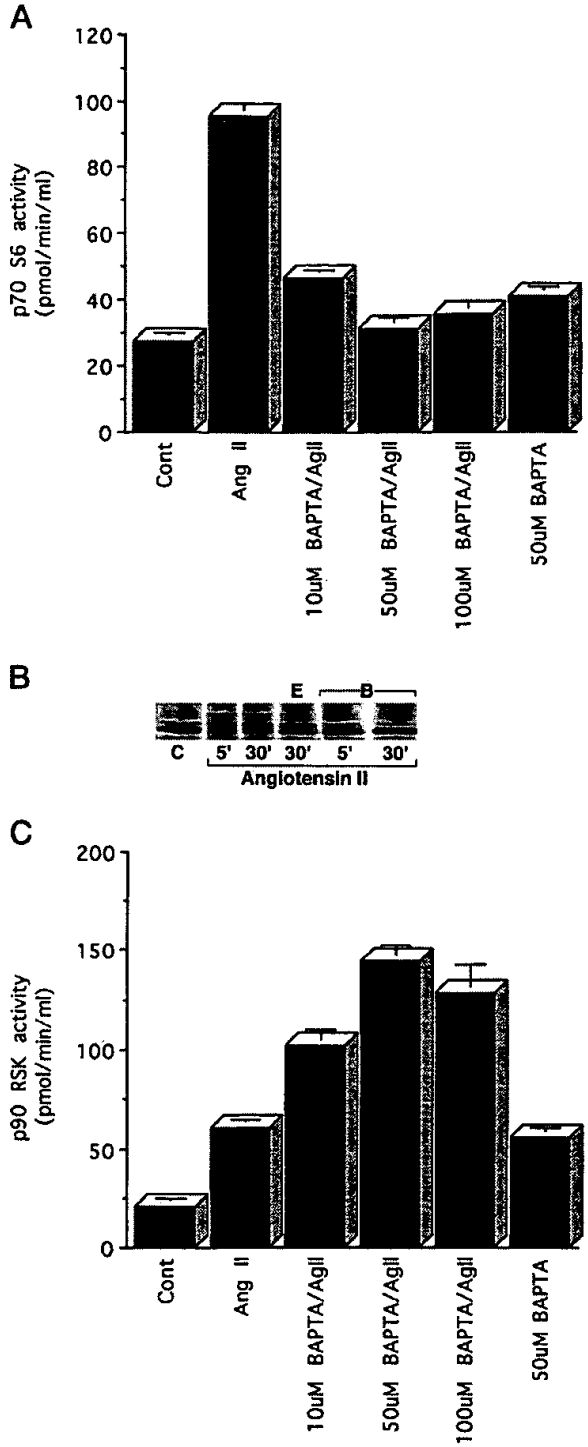

FIG. 5. **Angiotensin II activates $p70^{S6K}$ in a calcium-dependent manner.** *A*, $p70^{S6K}$ activity. Serum-starved GN4 cells were incubated with 1 μM Ang II for 20 min; in some experiments cells were incubated with the indicated concentrations of BAPTA for 15 min prior to the addition of Ang II (*AgII*). Cell lysates were prepared, and the $p70^{S6K}$ was immunoprecipitated and assayed as described earlier. The $p70^{S6K}$ activity is plotted as pmol/min/ml, and the results represent the mean ± S.E. (*bars*) of duplicate samples. *B*, serum-starved GN4 cells were incubated with Me_2SO (carrier), 5 mM EGTA (*E*), or 50 μM BAPTA (*B*) for 15 min prior to the addition of Ang II (1 μM) for the amount of time indicated; $p70^{S6K}$ was analyzed for activity by SDS-PAGE immunoblotting as described earlier. *C*, $p90^{RSK}$ activity. Cells were treated exactly as described in *A*, and the $p90^{RSK}$ was immunoprecipitated and assayed as described earlier. The $p90^{RSK}$ activity is plotted as pmol/min/ml, and the results represent the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 3$ experiments. *Cont*, control.

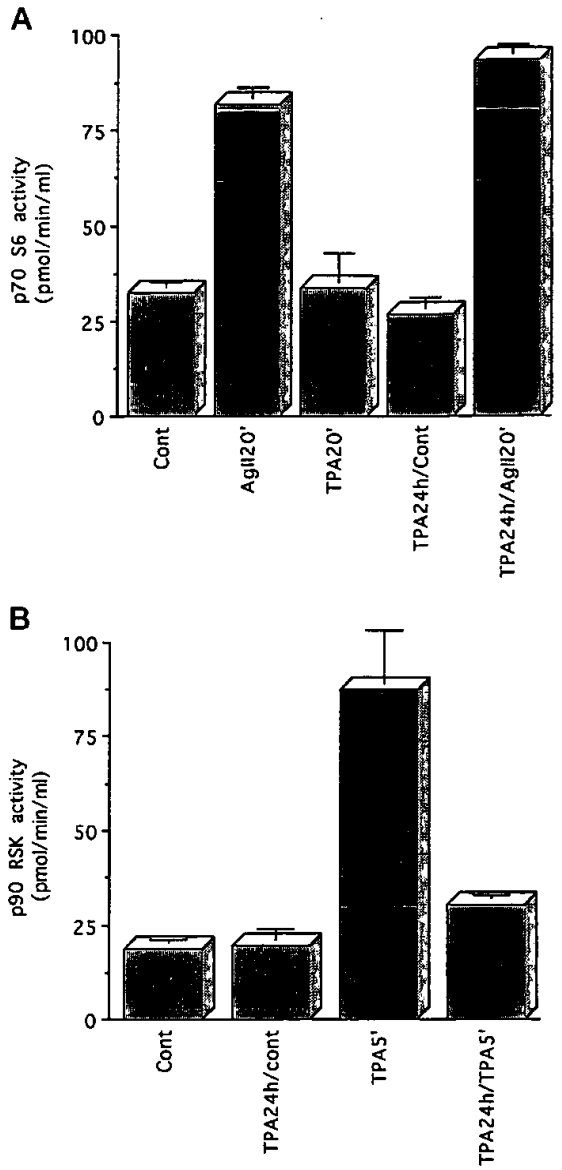

FIG. 6. **TPA activates p90^{RSK} but not p70^{S6K} in GN4 cells.** Serum-starved GN4 cells were stimulated with Ang II (*AgII*, 1 μM) or TPA (100 nM) for the amount of time indicated. In some experiments, cells were incubated with 1 μM TPA 24 h prior to collection. Cell lysates were prepared as described earlier and assayed for p70^{S6K} (*A*) or p90^{RSK} (*B*) activity. Kinase activity is plotted as pmol/min/ml, and the results represent the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of n = 4 experiments. *Cont*, control.

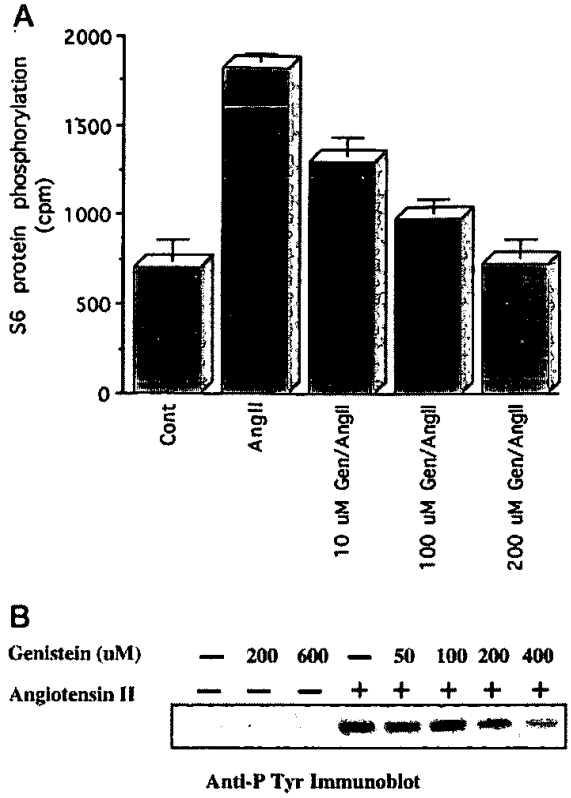

FIG. 7. **Genistein inhibits the activation of CADTK and p70^{S6K} by Ang II.** *A*, p70^{S6K} activity, serum-starved GN4 cells were incubated with Me_2SO (carrier for genistein) or genistein (*Gen*) for 15 min prior to the addition of Ang II (1 μM). After incubating cells with Ang II for 20 min, the cells were harvested, and the cell lysates were assayed for S6 kinase activity. The activity is plotted as radioactivity (cpm, ^{32}P) incorporated into the ribosomal S6 protein and represents the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of n = 3 experiments. *Cont*, control. *B*, CADTK, GN4 cells grown in 6-cm plates were serum-starved and incubated with the concentrations of genistein indicated for 15 min. After incubation with genistein, the cells were treated with 1 μM Ang II for 90 s, and the cells were washed twice with PBS and harvested in RIPA buffer (1 ml). The amount of tyrosine autophosphorylation on CADTK was determined by immunoprecipitation with CADTK-specific antisera followed by PT66 antiphosphotyrosine immunoblotting as described under "Experimental Procedures."

thelial cells (4), cloned the cDNA, and raised specific antisera to the protein (5). We compared the ability of genistein to inhibit the activity of CADTK by assaying the tyrosine autophosphorylation of CADTK, as described under "Experimental Procedures." The amount of tyrosine autophosphorylation of this kinase appears to be proportional to the enzymatic activity.[2] As shown in Fig. 7*B*, incubating GN4 cells with concentrations of genistein from 50 to 400 μM led to a progressive decrease in the Ang II-stimulated CADTK tyrosine autophosphorylation. Similar concentrations of genistein were required to inhibit the tyrosine autophosphorylation of the EGF receptor in these cells. In comparison, the calcium-dependent tyrosine phosphorylation of an exogenous substrate (*e.g.* paxillin, a 68–70-kDa protein) tyrosine-phosphorylated by CADTK was inhibited by even lower concentrations of genistein (100–200 μM) than was the CADTK autophosphorylation.[3] The results with genistein implicate a tyrosine kinase in the activation of p70^{S6K} by calcium or Ang II, suggesting a role for CADTK.

The Stimulation of p70^{S6K} Correlates with Increased CADTK Activity—To further investigate whether CADTK was involved in the regulation of p70^{S6K}, we compared the activation of p70^{S6K} in cells known to exhibit different levels of Ang II- and thapsigargin-dependent CADTK activation. Previously we showed that transformed GN4 rat liver epithelial cells exhibit approximately 3–4 times more Ang II-stimulated tyrosine phosphorylation than the parental cell type from which it was derived (WB) (3). We have recently confirmed that GN4 cells exhibit approximately 5 times more Ang II-dependent activation and 2–3-fold more CADTK protein than WB cells (5). Treatment with Ang II or the ionophore A23187 resulted in a significantly larger activation of p70^{S6K} in GN4 cells compared with WB cells (Fig. 8*A*). Typically, the ability of Ang II or A23187 to activate p70^{S6K} was at least 2–3-fold higher in GN4 cells than in WB cells and was independent of the length of

[2] L. Xiong, unpublished observations.

[3] L. Xiong, unpublished data.

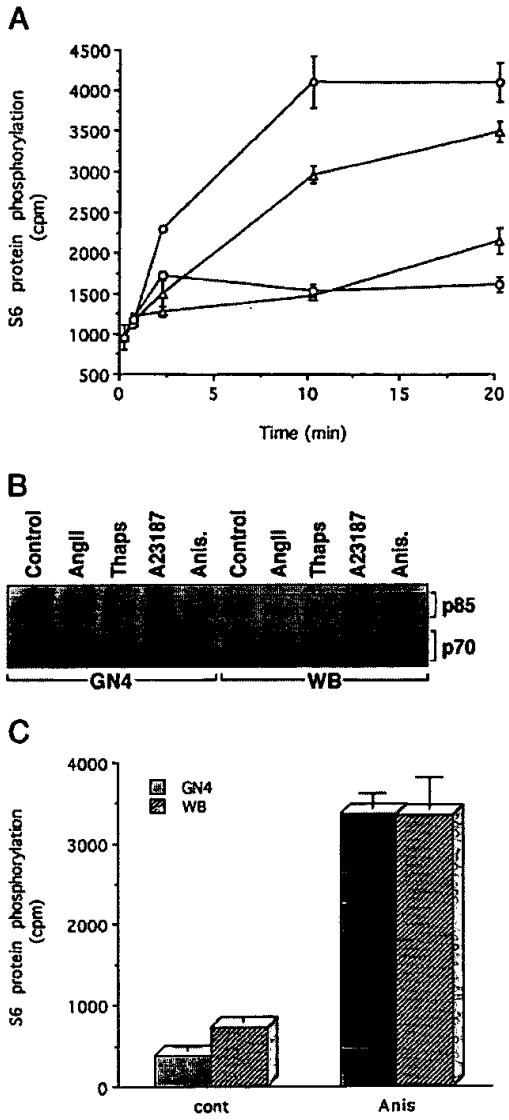

FIG. 8. **Increased expression of CADTK in GN4 cells correlates with increased activation of p70^{S6K} by calcium stimuli.** *A*, serum-starved GN4 cells were incubated with 1 μM A23187 (Δ) or 1 μM Ang II (O), and serum-starved WB cells were treated with 1 μM A23187 (△) or 1 μM Ang II (○) for the lengths of time indicated. The cells were washed twice with PBS, and the cell lysates were assayed for p70^{S6K} activity. The activity is plotted as radioactivity (cpm, ^{32}P) incorporated into the ribosomal S6 protein and represents the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 3$ experiments. *B*, GN4 and WB cells were stimulated with Ang II (1 μM), thapsigargin (*Thaps*, 2 μM), A23187 (1 μM), or anisomycin (*Anis.*, 10 μg/ml) for 20 min. Cell lysates were prepared, and the p70^{S6K} was detected by immunoprecipitation and immunoblotting as described earlier. *C*, GN4 (*solid bars*) and WB (*hatched bars*) cells were serum-starved and incubated with anisomycin (*Anis*, 10 μg/ml) for 20 min, after which the cells were harvested and assayed for p70^{S6K} activity as described earlier. The activity is plotted as radioactivity (cpm, ^{32}P) incorporated into the ribosomal S6 protein and represents the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 2$ experiments. *cont*, control.

exposure to these compounds. Similarly, thapsigargin stimulated a similar 2–3-fold higher increase in S6 kinase activity in GN4 cells (data not shown).

To eliminate the possibility that the enhanced calcium-dependent activation was simply due to increased expression of the p70^{S6K} in GN4 cells, the amount of p70^{S6K} activity and protein was compared in the two cell lines by immunoblotting and activity assays as described earlier. As determined by immunoblotting, the amount of the p70^{S6K} protein and the nuclear form of this kinase (p85^{S6K}) (33) was comparable in the two cell types (Fig. 8*B*). Importantly, in GN4 cells, Ang II, thapsigargin, and A23187 stimulated a pronounced mobility shift of both the p70 and p85 S6 kinases; by contrast, much less mobility shift was observed in WB cells, in agreement with the S6 kinase activity assays. Furthermore, the protein synthesis inhibitor anisomycin (20) strongly stimulated the mobility shift (Fig. 8*B*) and activity of p70^{S6K} (and p85^{S6K}) equivalently in WB and GN4 cells (Fig. 8*C*). These results demonstrate that differences in p70^{S6K} or p85^{S6K} expression do not account for the enhanced activation by calcium in GN4 cells. Instead these results suggest that the increased expression of CADTK in GN4 cells facilitates the activation of p70^{S6K} by Ang II or calcium stimuli.

DISCUSSION

Ang II has numerous effects on rat liver epithelial cells. It is a weak mitogen, a response that is initiated via the AT_1 receptor, transduced by G_q protein stimulation of phospholipase C, and effected by serine, threonine, and tyrosine phosphorylation (1). Elevating intracellular calcium with thapsigargin or ionophore (A23187) mimics the Ang II-stimulated increase in protein tyrosine phosphorylation in GN4 cells (2), and our laboratory has recently isolated a likely candidate for the calcium-stimulated entity that regulates tyrosine phosphorylation, a novel CADTK. Incubating GN4 cells with Ang II or agonists that raise intracellular calcium (*i.e.* thapsigargin and A23187) activates CADTK (4), whereas incubation with the calcium chelator BAPTA-AM inhibits activation of this enzyme. The effect of calcium on CADTK is indirect; adding calcium or calcium and calmodulin to cell lysates does not activate CADTK, and thus the mechanism by which calcium and other signals regulate this enzyme remains to be established.

In addition to activating PKC, the calcium- and calmodulin-dependent protein kinase, and CADTK in GN4 cells, Ang II also activates MAPK and JNK and increases AP-1 binding (the latter can be accomplished in a PKC-independent manner) (10). We now demonstrate Ang II and calcium-dependent activation of p70^{S6K}. The challenge is to discern which of these multiple Ang II-dependent pathways are downstream of PKC, calcium and calmodulin, CADTK, or even G-protein $\beta\gamma$ subunits.

We began this process by purifying CADTK; peptide and cDNA cloning has identified this enzyme as the rat homologue of a novel human nonreceptor tyrosine kinase, PYK-2 (4–6). Using PC12 cells, PYK-2 was shown to be activated by elevating intracellular calcium or PKC activity. In these cells, PYK-2 stimulation increased MAPK activity, providing a potential mechanism for calcium-dependent regulation of MAPK in these and other cells (6, 52). In contrast, our studies in rat epithelial cells demonstrate that a calcium signal (*i.e.* thapsigargin and A23187) does not significantly activate MAPK and that Ang II-dependent MAPK activation is primarily a PKC-dependent process.[2] Instead, Ang II and a calcium signal (thapsigargin) substantially activate JNK in a calcium-dependent manner (10). In fact, the Ang II effect is PKC-independent and is amplified in cells depleted of PKC. JNK activation by Ang II and thapsigargin correlates with increased expression and tyrosine autophosphorylation of CADTK in our rat liver epithelial cells. We have shown that the chemically transformed GN4 cell line expresses more CADTK than the parental cell line (WB), and the activation of CADTK by Ang II is approximately 5-fold greater in confluent GN4 cells when compared with WB cells. Activation of JNK by Ang II and thapsigargin is also 5-fold higher in GN4 cells, whereas the total JNK activity induced by stress (*e.g.* anisomycin) is equal in WB and GN4 cells. Thus in GN4 cells, CADTK is not involved in MAPK activation but is likely to be a mediator of signaling to JNK.

Because some of the stimuli known to activate JNK also increase $p70^{S6K}$ activity in other cell types, (*i.e.* Ang II (11) and anisomycin (20)), we compared the ability of a calcium signal to regulate $p70^{S6K}$ in epithelial cells. In this study we find that like JNK (10), $p70^{S6K}$ is regulated by agonists that raise intracellular calcium, whereas neither $p90^{RSK}$ nor MAPK is affected by the calcium-activated pathway. This is contrasted to results in PC12 cells (6, 52) and suggests that the ability of calcium to regulate these signaling pathways is cell type-specific.

The role of intracellular calcium and Ang II (through its effects on intracellular calcium) in stimulating $p70^{S6K}$ has not been studied extensively. In one article, A23187 weakly activated $p70^{S6K}$ in Swiss 3T3 fibroblasts (53), a cell line that does not appear to contain CADTK.[2] Earlier, the work of Meier *et al.* (54) reported the activation of an S6 kinase in kidney epithelial cells in response to calcium stimuli (A23187), although the identity of this kinase was not established. It is clear that in this study we were measuring the calcium-dependent activation of $p70^{S6K}$ in cell lysates. First, although the cell lysate assay could detect $p90^{RSK}$ phosphorylation of the 40 S ribosomes, we have used a specific immune complex assay to show that there is little if any thapsigargin-dependent $p90^{RSK}$ activation. Second, all our assay data have been confirmed by immunoblot analysis of the $p70^{S6K}$ mobility shift, an assay that highly correlates with the activation. Third, rapamycin, which inhibits $p70^{S6K}$ but not $p90^{RSK}$ activation, abolished the Ang II- or thapsigargin-dependent activation of 40 S ribosome phosphorylation in cell lysates.

Importantly, our results suggest that most of the Ang II-dependent activation of $p70^{S6K}$ is calcium-dependent. Preincubation with BAPTA prevented the Ang II-dependent activation of $p70^{S6K}$ but not $p90^{RSK}$. Instead, $p90^{RSK}$ and MAPK were substantially activated by BAPTA, alone or in the presence of Ang II. At this point, the mechanism of the BAPTA effect is unclear; we can only speculate that calcium may regulate one or more phosphatases in the MAPK cascade or that BAPTA has a non-calcium-dependent effect on the MAPK pathway. We also cannot exclude that additional G-protein-coupled signals, *e.g.* $\beta\gamma$ subunit or other G-protein-dependent responses, contribute to the stimulation of $p70^{S6K}$ by Ang II. Recently the activation of $p70^{S6K}$ by receptors coupled to G_i has been described, supporting a potential role for $\beta\gamma$ subunits in the regulation of $p70^{S6K}$ (55).

In this study we also found that agonists that increase intracellular calcium (A23187, thapsigargin, and Ang II) in GN4 cells activate $p70^{S6K}$ following a rapid stimulation of CADTK. The cell-permeable chelator BAPTA-AM inhibited the activation of both CADTK and $p70^{S6K}$ by thapsigargin, A23187, or Ang II, consistent with a role for CADTK upstream of $p70^{S6K}$ in these cells. In further support of a role for CADTK, the calcium- and Ang II-dependent activation of $p70^{S6K}$ was prevented by genistein at concentrations similar to those required to inhibit the thapsigargin-dependent JNK activation and the thapsigargin- and Ang II-dependent tyrosine phosphorylation of CADTK substrates (*e.g.* paxillin) *in vivo*.[2] Other findings linking CADTK to the $p70^{S6K}$ pathway come from a comparison of $p70^{S6K}$ activity in WB and GN4 cells. The GN4 cell line expresses 2–3-fold greater CADTK and has 5-fold greater Ang-II-dependent CADTK and JNK activation (5). GN4 cells also exhibit 2–3-fold greater $p70^{S6K}$ activation in response to Ang II and thapsigargin. The failure of $p70^{S6K}$ to match the 5-fold difference in CADTK and JNK activation between GN4 and WB cells may well stem from the complex pathway to $p70^{S6K}$, which appears to involve regulation of at least two upstream kinase pathways (see below).

One possibility is that a calcium signal may be activating PI-3 kinase, since low concentrations of wortmannin (50 nM) inhibited the activation of $p70^{S6K}$ by A23187, thapsigargin, or Ang II. Numerous studies have suggested that PI-3 kinase is an upstream mediator of $p70^{S6K}$ activity (reviewed in Ref. 12), although much of the evidence for PI-3 kinase (including our study) has been obtained with the inhibitor wortmannin (48), the selectivity of which has been recently questioned (56). With the caveat of specificity, the activation of CADTK by Ang II or thapsigargin is not inhibited by wortmannin,[2] suggesting that PI-3 kinase is downstream or unrelated to the calcium-dependent activation of CADTK. Wortmannin also does not inhibit JNK activation, another pathway linked to the regulation of CADTK in GN4 cells.[2] Thus, if CADTK is a common activator of both $p70^{S6K}$ and JNK in GN4 cells, as our results suggest, at some point the signaling pathways diverge such that a wortmannin-sensitive step is specific to the $p70^{S6K}$ pathway.

Our results are also consistent with the calcium-dependent activation of another limb of the $p70^{S6K}$ pathway, the one that activates the rapamycin-sensitive kinase, *i.e.* FRAP/RAFT. Rapamycin inhibits the activity of the FRAP/RAFT kinases by binding to its cognate binding protein, FKB12, thus inhibiting $p70^{S6K}$ stimulation (16). However, like the PI-3 kinases, the role of FRAP/RAFT in regulating $p70^{S6K}$ is poorly understood, largely because substrates for these kinases remain to be identified. Again the calcium-dependent pathway to JNK and $p70^{S6K}$ is divergent in GN4 cells, since rapamycin blocks Ang II- and thapsigargin-dependent $p70^{S6K}$ activation but does not effect the stimulation of CADTK or JNK by these agonists.[2]

In addition to increasing intracellular calcium, Ang II also stimulates PKC activity (1, 5). Despite the fact that PKC has been shown to activate $p70^{S6K}$ in other cell types (57), our results do not support a role for PKC in the regulation of $p70^{S6K}$ in GN4 cells. Although Ang II stimulated $p70^{S6K}$ in a manner similar to thapsigargin or A23187, TPA alone did not increase $p70^{S6K}$ activity in GN4 cells, although MAPK and $p90^{RSK}$ were activated as expected.

TPA also increases CADTK activity in these cells, albeit less potently than Ang II and more slowly than thapsigargin. These results present a paradox, since our studies suggest that CADTK is upstream of $p70^{S6K}$, and yet TPA does not activate $p70^{S6K}$. We have found that a brief (5 min) TPA pretreatment inhibited the activation of $p70^{S6K}$ by Ang II or thapsigargin by approximately 40% in GN4 cells.[4] Similarly, an even more profound inhibitory effect of PKC has been found on the activation of JNK by calcium stimuli or Ang II in GN4 cells.[2] Taken together, these studies demonstrate that PKC alone is incapable of activating $p70^{S6K}$ and may inhibit the downstream calcium-dependent signals to both JNK and $p70^{S6K}$ in GN4 cells.

In summary, we have demonstrated a calcium-dependent pathway that results in substantial activation of $p70^{S6K}$. This calcium-dependent pathway has characteristics similar to to the calcium-dependent pathway to JNK that we have previously studied in this cell line (5, 10). Intriguingly, this calcium-dependent pathway does not activate MAPK or $p90^{RSK}$; instead these enzymes are activated in PKC-dependent manner. Considerable circumstantial evidence points to CADTK as an initial mediator of the calcium signal to both JNK and $p70^{S6K}$. However, the pathway to these enzymes clearly diverges at some point such that the calcium-dependent activation of JNK is insensitive to wortmannin and rapamycin, whereas the activation of $p70^{S6K}$ is completely inhibited by these compounds. The exact signaling downstream from CADTK and, in fact, unequivocal proof of CADTK involvement must await further studies.

[4] L. M. Graves, unpublished observations.

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